PROGRESS REPORT

RESEARCH ON PROCEDURES FOR THE

LOW-TEMPERATURE PRESERVATION OF BLOOD

XI

CONTRACT NO. Nonr-3003(00)

PREPARED FOR

OFFICE OF NAVAL RESEARCH

DEPARTMENT OF THE NAVY

UNION CARBIDE

LINDE COMPANY

DIVISION OF UNION CARBIDE CORPORATION

RESEARCH LABORATORY

TONAWANDA, NEW YORK

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DEVELOPMENT AND CLINICAL EVALUATION OF PROCESSES FOR THE LOW TEMPERATURE PRESERVATION OF BLOOD BASED ON RAPID COOLING AND WARMING AND PROTECTION BY POLYMERS

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Submitted by

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PROGRESS REPORT ON CONTRACT NONR-3003(00) XI

Development and Clinical Evaluation of Processes for the Low Temperature Preservation of Blood Based on Rapid Cooling and Warming and Protection by Polymers

I. SCOPE OF THE REPORT

This report describes research and development studies from approximately February, 1961, to June, 1962, directed toward providing practical processes for the low temperature preservation of human blood by rapid cooling and warming and protection by extracellular polymeric solutes. Results of red cell survival studies in animal and human subjects are presented as are the data obtained in purely laboratory work.

A description of four basic processes formulated in this work and subjected to evaluation in rabbits and man is presented. These involve, in each case, minimum or no processing of the blood after thawing. A transfusion grade of the plasma expander, polyvinylpyrrolidone, has proven to be the most effective of a variety of polymeric additives evaluated.

II. SUMMARY

On the basis of previous observations in our laboratory, rapid freezing and thawing in combination with the use of extracellular, polymeric, protective additives appeared to offer the greatest potential of preserving blood at low temperature with minimum or no requirements for post-thaw processing. Four basic processes have been formulated and evaluated at a laboratory level and in animal and human transfusions.

Process I involves separating the red cells from blood by conventional centrifugation, resuspending the red cells and freezing in a polymer containing medium, and after thawing resuspending the cells in their plasma for transfusion. Polyvinylpyrrolidone (PVP) has been studied extensively in this process.

Process II involves freezing the red cell fraction of blood in the presence of part or all of the plasma combined with polymeric additives. PVP has been employed as the additive. After thawing the red cells are separated and resuspended in plasma or a plasma substitute.

Process III involves freezing whole blood collected into or combined after collection with polyvinylpyrrolidone at a concentration of about 7% w/v. At this concentration of PVP quantities of frozen blood up to 400 ml have been transfused to human recipients without post-thaw processing of any kind. No adverse clinical effects have been observed.

Process IV involves freezing the red cell fraction of blood in the presence of a small volume (about 25% of the cell volume) of polyvinyl-pyrrolidone-plasma or human serum albumin. After thawing the mixture is diluted by addition of isotonic saline or plasma to reduce the hematocrit and additive concentration. No post-thaw centrifuging or other handling is involved.

Table II-1 summarizes in vitro recoveries and in vivo survivals of preserved and control erythrocytes observed in over a hundred small volume experiments. Transfusion volumes of 400 ml, or less, have been used with blood frozen by Processes I and III without incident and with essentially similar results to those obtained with small volumes.

TABLE II-1

SUMMARY OF AVERAGE VALUES OBTAINED IN CLINICAL STUDY OF PROCESSES

	No. of Tests	In Vitro RBC Recovery %	In Vivo RBC Survival, % 1/2 Hr.	24 Hr.
Process I	28	90(93) (a)	90	74
Process II	25	96(97) (b)	89	74
Process III*	9	97	92	83
Process III**	4	97	98	84
Process IV	9	90	90	71
	17*	--	103	97
Controls	16	--	95	90
	10	--	99	91

* Data on 1/2 pint volumes
** Data on 1 pint volumes - best preservation procedure
(a) After resuspension in autologous plasma
(b) After resuspension in 6% dextran

III. INTRODUCTION

For the past three years the Linde Company, under contract to the Office of Naval Research, has studied the problem of preserving blood in the frozen state. The prime objective has been, and remains, a procedure suited to field use by the armed forces. This does not exclude applicability to civilian needs for long-term blood preservation, but implies that processing techniques be simple and rapid in execution, an obvious advantage to both categories of use. For military purposes the stricture has been imposed that no processing be required between the conclusion of the thawing step and the start of transfusion. This means that compounds combined with blood, to provide protection to the erythrocytes during freezing and thawing, must be acceptable as transfusible substances. At the same time, they must maintain to the highest attainable degree the osmotic stability, the viability and the functionality of the preserved cells. Further, they must protect a very high percentage of the red cells from lysis during the preservation process, so that the amount of free hemoglobin and cellular debris does not exceed tolerable limits during transfusion of multiple units of preserved blood.

These requirements have compelled a painstaking, step-by-step analysis of a multiplicity of factors, ranging from the physical and biological through areas in which there are various degrees of physical and biological interrelationship. The information obtained has been submitted in ten previous reports to the Office of Naval Research. This earlier work has led to the development of rapid low-temperature blood processing techniques. Additive substances such as lactose and glucose, which appear to protect red cells during freezing and thawing, have been studied and rejected as suitable agents to be combined with whole blood and transfused. Prior studies in our laboratory and elsewhere (1, 2, 3, 4, 5) have established the protective action of polymers in the preservation of whole blood and red cells by freezing. Such substances do not penetrate the cell membrane and can thus be rapidly removed by simple centrifuging or left in the preserved blood without risk of large scale osmotic lysis during transfusion.

These findings have enabled us to devise four processes suited to the use of polymeric additives. Various properties of blood preserved with these processes are described herein. In vitro experimentation and in vivo animal studies were carried out at our laboratories in Tonawanda, New York. Investigations involving the use of human subjects were carried out at the Veterans Administration Hospital in Buffalo under the direction of Dr. Marvin L. Bloom and Dr. Ernest Witebsky of the University of Buffalo, Medical School. Studies designed to perfect techniques for in vivo assay of preserved human blood were also carried out at the Roswell Park Memorial Institute, Buffalo, under the direction of Dr. Raymond S. Kibler. Preliminary evaluation of blood preserved by Processes II and IV was carried out by Dr. Kibler. The data on preserved human red cell survival are presented in Section VI of this report.

IV. LABORATORY RESULTS

In this section we report the results of in vitro experimentation of both a research and development nature which has preceded and paralleled studies in animal and human subjects. Chronologically, whole blood modified by addition of polymers was first investigated. Other processes evolved as we sought to minimize the amount of additive to be transfused especially for initial clinical testing.

Results are discussed in terms of chemical and physical parameters, that is, the composition of the preparation subjected to freezing and thawing and the physical conditions of cooling and warming. In addition, large volume (full pint) processing results are discussed and flow sheets defining each basic process are included.

Methods

Laboratory studies have in general been carried out on 50 ml volumes of blood or red cell suspensions. Freezing and thawing has been done, unless otherwise stated, under conditions of mechanical agitation using the Blood Processing Unit (BPU-1) described in ONR Progress Report VII (1961)[6]. Liquid nitrogen has been employed as the refrigerant and water as the medium for thawing. Laboratory, animal, and small volume clinical studies have involved processing aluminum containers of rectangular cross-section, 19 mm wide. (Figure IV-1).

For processes I, II, and IV red cells were separated from blood and suspended in suitable volumes and compositions of polymer-containing media. For Process III whole blood was combined with concentrated polymer solutions to give final concentrations desired.

Direct red cell recoveries have been determined after thawing by use of the sample hematocrit and colorimetric measurements of free and total hemoglobin concentrations.

Resuspension red cell recoveries have been similarly determined after diluting the red cells 1:2 in plasma, 6% dextran, etc. or 1:100 in unbuffered isotonic (0.15 M NaCl) sodium chloride or buffered isotonic 3.5% polyvinylpyrrolidone.

Efficiencies of Process (EOPs) refer to red cell recoveries after all losses during freezing, thawing, and resuspension are taken into account. Direct recovery times resuspension recovery equals efficiency of process.

In order to avoid misinterpretation of the latter term, it is emphasized that in the present report efficiency of process refers to in vitro results only.

1. Process I: Red Cells in Polymer Medium

It is well established that both the composition of the suspending medium and the conditions of cooling and warming influence the injury and protection of red cells during freezing and thawing.[1] Using artificial media of defined composition it is possible to study the effects of each and all constituents in which the red cells are suspended during processing. Moreover, the autologous plasma is available for subsequent resuspension of the thawed red cells. Their in vivo viability can be estimated under conditions equivalent to whole blood control specimens.

Using red cells suspended during freezing and thawing in defined media eliminates many restrictions on composition. Optimum chemical conditions can be readily sought for minimizing injury. The use of extracellular polymeric protective solutes allows rapid removal after thawing and immediate resuspension without osmotic problems inherent with small molecular weight solutes. Simple conventional centrifuging suffices to separate the red cells from their suspending medium and washing is not necessary.

Methods

For most of our experimental work volumes of red cell suspensions of 30 to 90 ml (average 50 ml) were frozen and thawed in aluminum containers 19 mm thick and of rectangular cross-section. Shell freezing was employed, this being accomplished by mechanical agitation at controlled frequencies in liquid nitrogen. Cooling rates were varied by means of volume and the use of appropriate heat transfer promoting coatings on the outside surfaces of the containers. A typical laboratory container is shown in Figure IV-1.

Thawing was accomplished by mechanical agitation (to promote convective heat transfer) in warm water. Conditions for particular experiments are described in footnotes to each table or figure.

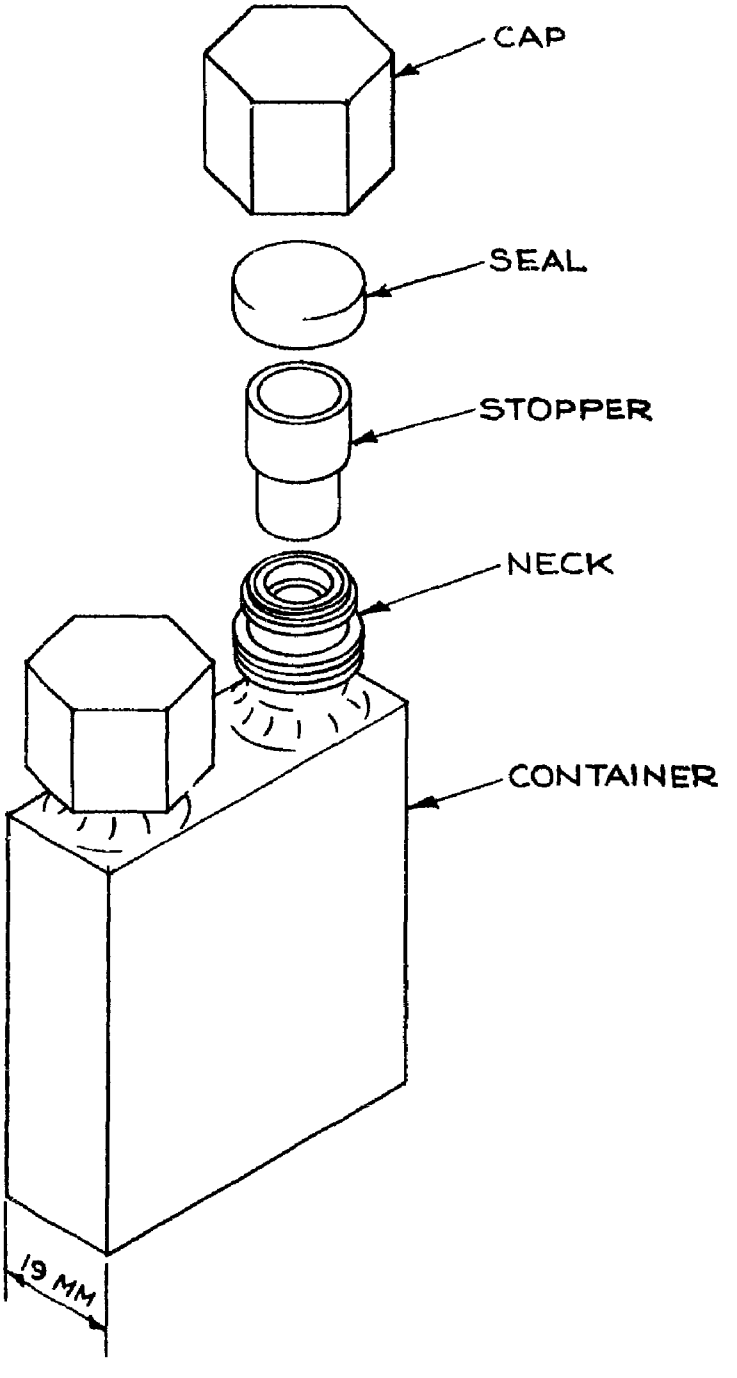
For experiments described in this section (i.e., Process I) whole blood-ACD was separated by centrifuging into the cellular and plasma fractions. The red cells were resuspended in polymer solutions and subjected to freezing and thawing. Direct recoveries and resuspension recoveries of red cells were estimated as described above.

A. CHEMICAL PARAMETERS

Polyvinylpyrrolidone K-30 (average M.W. 40,000) has been studied most extensively since preliminary small volume tests indicated its superiority over a variety of other polymers including dextran, gelatin, oxypolygelatin, low molecular weight (M.W. 10,000) polyvinylpyrrolidone and others. Table IV-1 shows the results of varying PVP concentration in a system

Figure IV-1

Experimental Container for Small Scale Processing
of Blood by Freezing



containing red cells suspended in an equal volume of PVP in isotonic saline. Similar studies using K-15 (M. W. 10,000) PVP gave recoveries ranging from 69-95% over the same concentration range. Direct recoveries increased continuously with concentration, however, resuspension stability passed through a distinct optimum.

TABLE IV-1

EFFECT OF CONCENTRATIONS OF

POLYVINYLPYRROLIDONE ON RED CELL RECOVERY

PVP K30 Conc. %	Direct % RBC Recovery	% Resuspension Recovery		
		Plasma	6% Dextran	5% Albumin
10	79	56	83	49
20	94	90	94	84
30	96	95	97	93
40	98	--	--	--
50	98	83	88	88
60	99	65	57	54

RBCS in equal volume PVP K30 in 0.15 M NaCl.
Frozen in BFF - 1975 container (40 ml) using PVP-MeOH coating, 200 cpm agitation. Thawed at 45°, 200 cpm.

Salt concentration influenced direct and resuspension recoveries. Both the absence of salt and its presence at 0.15 M were less favorable than an intermediate low concentration (Table IV-2).

Polyvinylpyrrolidones of K-values ranging from about 12 to 30 were examined. The average molecular weights corresponding to various K-values are shown in Figure IV-2. Molecular weights above about 10,000 were required for optimum red cell recovery. A slight drop in resuspension stability was observed at high molecular weight (above K-22, M.W. 25,000). (Figure IV-3).

TABLE IV-2

EFFECT OF CONCENTRATIONS OF POLYVINYLPYRROLIDONE

AND SALT ON RED CELL RECOVERY

PVP K-30 Conc. w/v%	Salt Conc. w/v%	Direct % RBC Recovery	Resuspension Recovery % Plasma	Wash	Plasma
10	0	74	88	81	93
10	0.3	86	91	83	92
10	0.9	83	77	54	89
15	0	77	91	83	95
15	0.3	90	95	91	95
15	0.9	90	92	76	92

RBCs suspended in equal volume of PVP K-30 - salt solution and frozen in BFF-19110 containers with PVP-Methanol coating in liquid nitrogen with mechanical agitation (2-1/2 inch amplitude, 200 cycles/minute). Thawing was done under same agitation conditions in 45°C water.

Thawed cells were separated and suspended in autologous plasma; separate aliquot was washed with buffered saline containing 0.4% glucose and resuspended in plasma.

Figure IV - 2

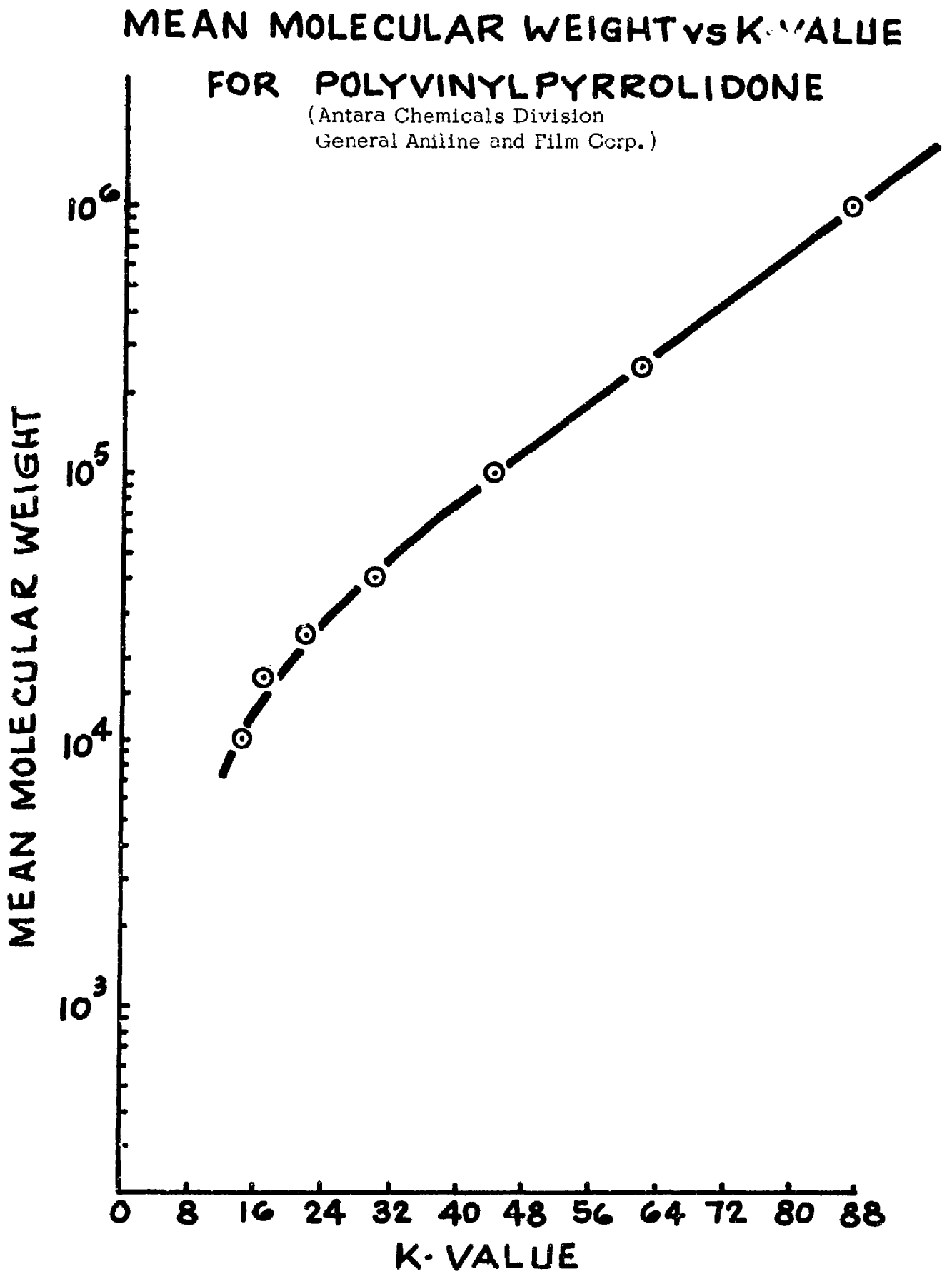
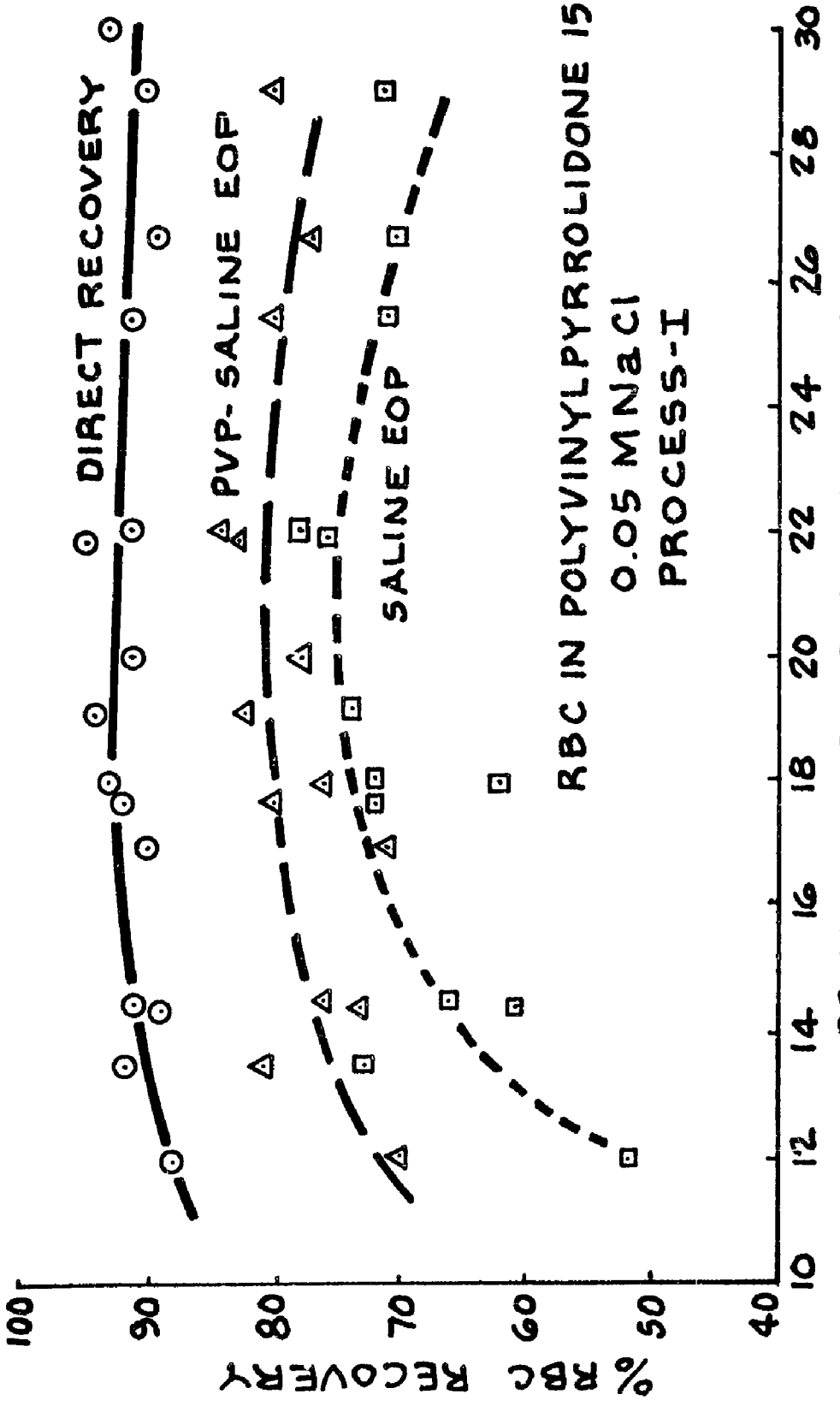

MEAN MOLECULAR WEIGHT vs K-VALUE
FOR POLYVINYLPYRROLIDONE
(Antara Chemicals Division
General Aniline and Film Corp.)
MEAN MOLECULAR WEIGHT
10^6
10^5
10^4
10^3
0
8
16
24
32
40
48
56
64
72
80
88
K-VALUE

Figure IV-3

Red Cell Recovery as a Function of Molecular Weight of Polyvinylpyrrolidone



(Molecular Weights Expressed in Terms of K-values, see Figure IV-2).

B. PHYSICAL PARAMETERS

In contrast to findings previously reported for protection of red cells by sugars, cooling conditions attained with silica-glycerol heat-transfer-promoting coatings appear to be too rapid for red cells in polymer medium. Representative results are plotted in Figure IV-4 as red cell recoveries versus A/VR* values (see Appendix A). Optimum heat transfer conditions were obtained by coating the metal container with a solution of polyvinylpyrrolidone K-30 in methanol (500 centipoise viscosity).

Thawing by mechanical agitation in water at 55° improved in vitro direct and resuspension recoveries by several per cent as compared with results obtained at 45° C.

Variation in specimen volume from 10 to 60% of container capacity indicated that red cell recovery was sensitive to volume only below about 30%. This could be ascribed to excessively rapid cooling or altered formation of the frozen shell during cooling.

Plastic containers were inferior to metal in terms of direct recovery. Resuspension stability was similar (Table IV-3).

TABLE IV-3

COMPARISON OF METAL AND PLASTIC CONTAINERS

FOR PROCESS I (PVP K-30 20%-0.05 M NaCl)

Container	Direct % RBC Recovery	Resuspension Recovery % Saline	PVP
Metal [(a)]	97	87	92
Plastic [(b)]	91	87	91

(a) BFF-1975 aluminum containers of rectangular cross-section 64 x 64 x 19 mm. Capacity 75 ml.

(b) Low density polyethylene bottles of rectangular cross-section 52 x 60 x 24 mm. Capacity 65 ml.

Each type container filled to 45% of capacity and frozen with mechanical agitation in liquid nitrogen and thawed in 45° C water.

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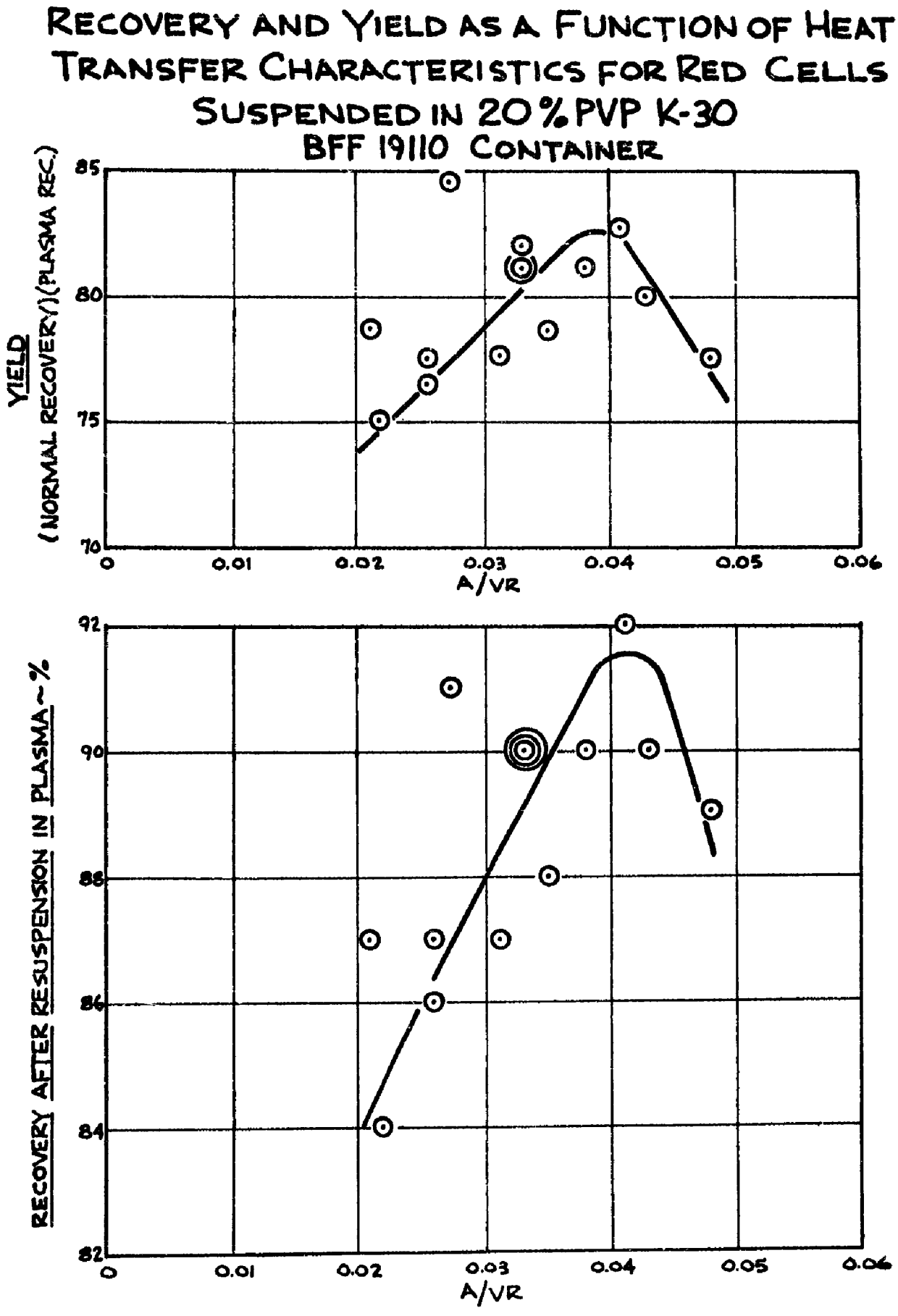
*See Progress Report III, December 15, 1960, for the derivation of A/VR.

Figure IV-4


RECOVERY AND YIELD AS A FUNCTION OF HEAT TRANSFER CHARACTERISTICS FOR RED CELLS SUSPENDED IN 20% PVP K-30
BFF 19110 CONTAINER
YIELD
(NORMAL RECOVERY)(PLASMA REC.)
85
80
75
70
0
0.01
0.02
0.03
0.04
0.05
0.06
A/VR
RECOVERY AFTER RESUSPENSION IN PLASMA ~ %
92
90
88
86
84
82
0
0.01
0.02
0.03
0.04
0.05
0.06
A/VR

C. PROCESS DEVELOPMENT

Figure IV-5 gives a simple flow outline for Process I. Full pint volumes of ACD-B blood collected in bottles or plastic packs have been processed under simulated (and as described in Section VI, actual) aseptic conditions with recoveries of red cells of 94% or better.

Table IV-4 shows results obtained in eight experiments using 15% PVP-0.05 M NaCl as the medium. Table IV-5 shows the variations in composition of the medium. Essentially equivalent results have been obtained in bottles and in plastic bags (Table IV-6). The use of plastic packs allows considerable improvement in processing time since centrifugation can be done at high speed. Approximately 15-20 minutes are required to separate the thawed cells.

2. Process II : Red Cells in Plasma-Polymer Medium

In attempts to improve the red cell recovery for Process I the ratio of red cells to PVP suspending medium was varied. Included among the samples studied were some in which red cells were suspended in a medium composed of plasma and polyvinylpyrrolidone solution. As shown in Table IV-7 improvement in red cell direct and resuspension recoveries resulted when plasma was present.

When the amount of plasma was varied from 0 to 70% of the medium (by volume) at constant PVP concentration (15%), direct red cell recovery was observed to vary only slightly, while a marked improvement in saline and PVP (isotonic-isooncotic) resuspension stability resulted (Figure IV-6). Above a level of 50% plasma, no improvement was found.

These observations suggested a process in which only a part of the plasma would be removed from whole blood prior to combining with a protective additive solution. This method has been designated Process II .

A. CHEMICAL PARAMETERS

An optimum concentration of PVP (K-30) between 15 and 30% was found for Process II when 50% plasma was present (Table IV-8). Using either K-30 (average M.W. 40,000) or K-22 (average M. W. 25,000) polyvinylpyrrolidone at a concentration (w/v) of 20% essentially equivalent recoveries and resuspension stabilities were obtained over the range of plasma concentrations 15 to 60% (by volume) (Figure IV-7).

Preparations of polyvinylpyrrolidone varying in K-value from 12 to 30 (M.W. 8,000 to 40,000) have been provided by Antara Chemicals Division of General Aniline and Film Corporation. Figure IV-8 illustrates the essential

FIGURE IV - 5

FLOW SHEET FOR PROCESS - i

PRESERVATION OF RED CELLS IN POLYMER MEDIUM

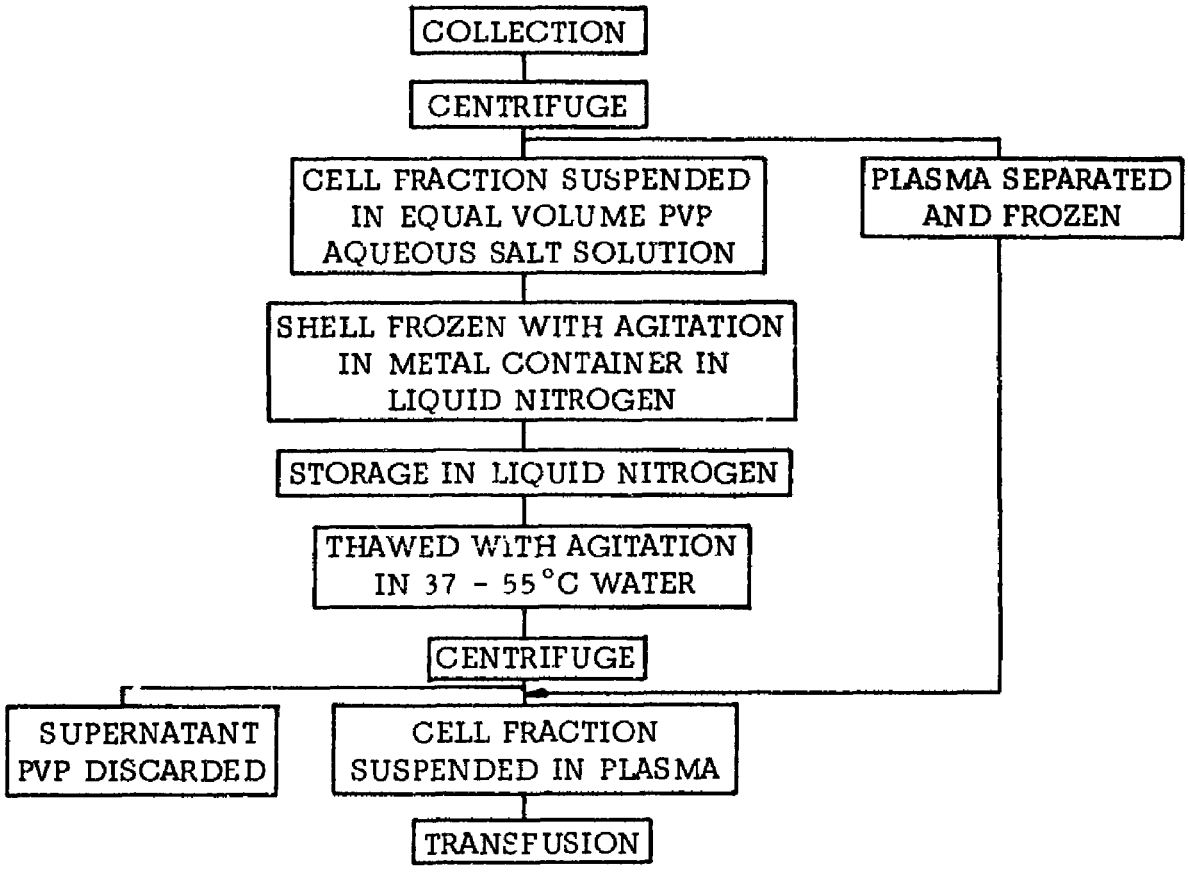


TABLE IV-4

PROCESSING OF PINT VOLUMES OF

BLOOD BY PROCESS I

Exp. No	% Direct RBC Recovery	% Resuspension Recovery Dextran[a]	Plasma[a]
1	92	96	94
2	88	---	93
3	93	95	93
4	90	95	92
5	91	96	94
6	90	95	92
7	93	97	93
8	92	96	94

RBCs suspended in equal volume 15% K-30 PVP-0.05 M NaCl. Frozen in pint container (capacity 1100 ml approximately) with PVP-MeOH coating, 200 cpm agitation. Thawed 25 seconds, 200 cpm, in 55° water.

(a) Resuspension of RBCs 1:2 in autologous plasma or 6% Dextran (Baxter Gentran M.W. 72,000 approximately).

TABLE IV-5

RED CELL RECOVERY DATA FOR

1 PINT PROCESS I TESTS

Additive Composition PVP (%)	NaCl (M)	Glucose (%)	Number of Tests	%INTACT CELLS Thawed Blood Direct RBC Recovery	Thawed Blood Saline* EOP	Reconstituted Blood Direct RBC Recovery	Reconstituted Blood Saline* EOP
20	.05	0	4**	90	81	95	88
20	.05	0	13	94	81	95	88
20	.075	0	1	96	82	96	84
20	.10	0	1	96	76	91	79
20	.15	0	3	94	70	88	71
15	.05	0	3	93	85	96	90
15	.075	0	1	95	77	93	79
20	.05	2	5	96	86	98	88

*1 volume of blood diluted 100-fold with physiological saline then allowed to stand 1/2 hour prior to analysis for free hemoglobin.

**Thawing at 200 cpm in 45°C water bath. Other samples were agitated at 150-160 cpm.

BLOOD FROZEN: 300 cc of additive per unit of packed cells from a pint collection.

FREEZING CONDITIONS: 200 cpm in liquid nitrogen using corrugated aluminum 1 pint containers.

TABLE IV-6

EFFECT OF ANTICOAGULANT ON BLOOD PRESERVATION WITH PROCESS I

Anticoagulant	[NaCl] in Additive (M)	% INTACT CELLS			
		Thawed Blood		Reconstituted Blood	
		Direct RBC Recovery	Saline EOP	Direct RBC Recovery	Saline EOP
ACD-A	.05	92.9	75	89.6	82.5
ACD-A	.1	94.9	75	89.9	79.3
ACD-A	.1	95.0	79.8	90.6	85.0
CPD	.1	93.9	74.5	87.7	78.7
ACD-A	.1	93.1	75.8	89.7	80.2
CPD	.1	92.9	72.6	86.3	76.7
ACD-B	.05	94.2	81.9	95.7	90.9
ACD-B	.05	94.1	80.6	95.5	90.4

Blood: Collected into Fenwal Blood Packs by Red Cross.

Additive: 20% Plasdone-C solution containing NaCl.
300 cc added to each unit of packed cells.

Containers: FC-1 uncoated

Thawing Conditions: 150 cpm agitation in 45°C water bath.

Reconstitution: Remove supernatant from packed cells after thawing and resuspend in autologous plasma.

Saline EOP: Dilute blood 100-fold with physiological saline and analyze for free hemoglobin after 1/2 hour standing at room temperature.

TABLE IV-7

EFFECT OF VARYING THE COMPOSITION
OF MEDIUM FOR PROCESS - I

Vol. RBCS	Vol. Plasma	Vol. PVP Soln.	Conc.% PVP Stock*	% RBC Recovery	% Resuspension Recovery	
					Saline	PVP
1	0	0.5	15	94	87	94
1	0	1.0	15	93	89	95
1	0	2.0	15	90	85	93
1	0.5	0.5	30	97	90	97
1	1.0	1.0	30	97	92	95

* K30 PVP in 0.05M NaCl.

53 ml of RBC - PVP or RBC-Plasma-PVP mixture frozen in BFF-19110 containers coated with PVP-MeOH by agitation at 200 cpm 2-1/2 inch amplitude in liquid nitrogen and thawed at 200 cpm in 45° water.

TABLE IV-8

EFFECT OF POLYVINYLPYRROLIDONE
CONCENTRATION IN PROCESS-II

K30 PVP Conc. w/v%	Direct % RBC Recovery	Resuspension Recovery %	
		Saline	PVP
10	93 ± 0	89 ± 0	91 ± 1
15	94 ± 1	94 ± 1	95 ± 0
20	95 ± 0	94 ± 0	95 ± 0
30	96 ± 1	90 ± 1	94 ± 0

RBC/Medium = 1:2 Medium: 50% Plasma -PVP - 0.1 M NaCl.

FIGURE IV-6

EFFECT OF PLASMA IN SUSPENDING MEDIUM

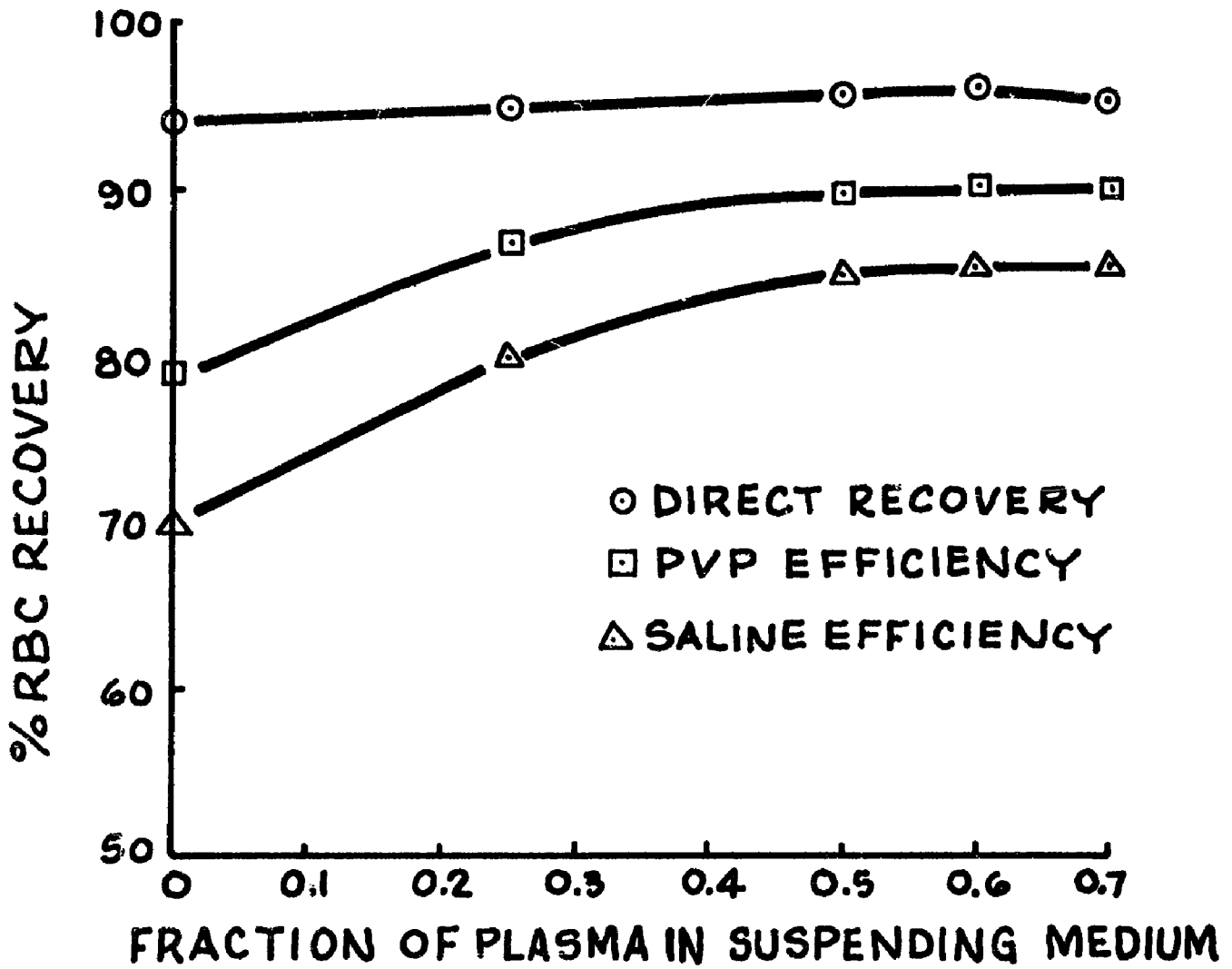


FIGURE IV-7

RED CELL RECOVERY AND RESUSPENSION STABILITY AS A FUNCTION OF PLASMA CONCENTRATION

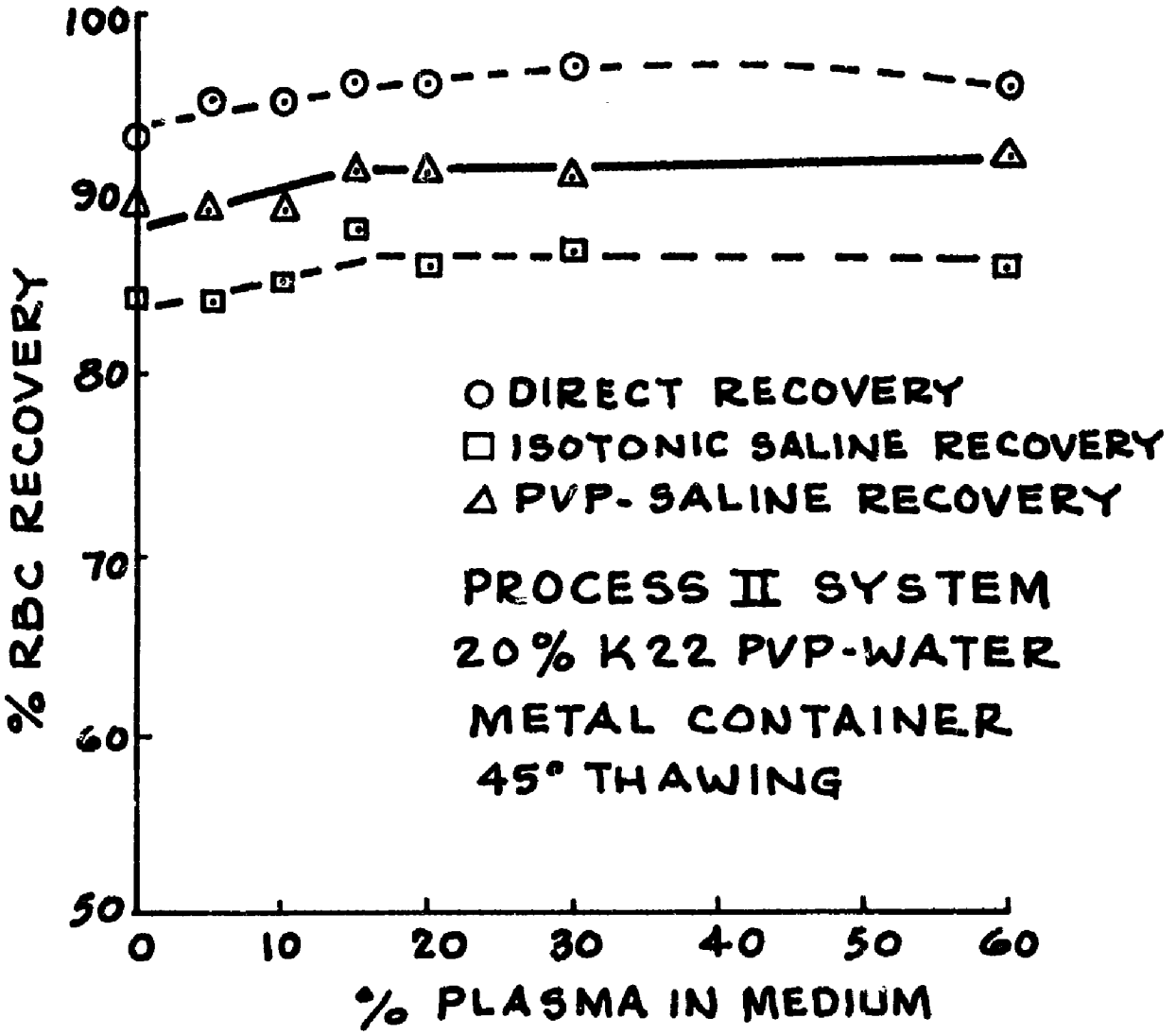
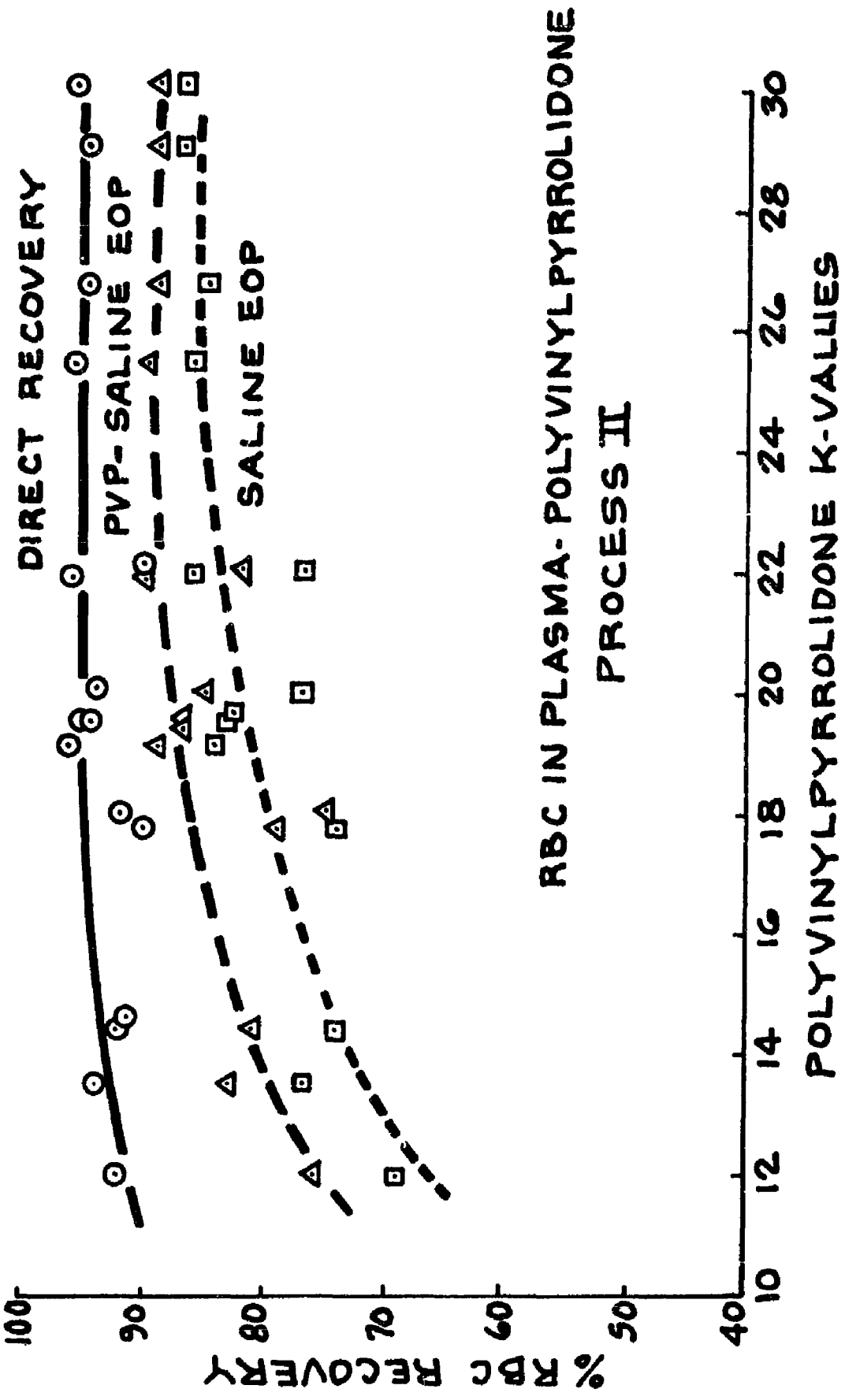


FIGURE IV-8


RBC IN PLASMA- POLYVINYLPYRROLIDONE
PROCESS II
DIRECT RECOVERY
PVP- SALINE EOP
SALINE EOP
% RBC RECOVERY
100
90
80
70
60
50
40
10
12
14
16
18
20
22
24
26
28
30
POLYVINYLPYRROLIDONE K-VALUES

equivalency of PVP's of molecular weights greater than about 25,000.

Varying the ratio of red cells to suspending medium improved direct red cell recoveries and resuspension recoveries. The presence of 1 to 2 volumes of medium (50% plasma-15% PVP) per volume of red cells gave recoveries significantly better than lesser volumes (Table IV-9). Larger volumes of suspending medium were equivalent but did not improve recoveries.

TABLE IV-9

EFFECT OF VOLUME RATIO BETWEEN

RED CELLS AND MEDIUM (PROCESS-II)

Vol. Medium Per Vol. RBCS	Direct % RBC Recovery	% Resuspension Recovery		
		Dextran (a)	Saline (b)	PVP (b)
0.25	94	95	86	93
0.5	95	96	88	93
1.0	94	97	94	96
2.0	95	98	94	97

RBC's separated from whole blood washed with plasma-PVP, and resuspended in volumes of medium shown per volume cells. Frozen with PVP-Methanol coating in BFF-19110 with mechanical agitation 200 cpm. Thawed at 45°C and 150 cpm.

(a) Dilution 1:2 (b) Dilution 1:100

Plasma, polyvinylpyrrolidone, and salt concentrations were studied simultaneously to define an optimum composition and delineate the useful limits of this system. Representative results are shown in Table IV-10. An optimum in PVP concentration of about 20% exists. Plasma concentration can be as low as 15-30% of the medium by volume. Salt appears to be injurious at 0.15 M but at lower concentrations can be varied considerably without affecting red cell recovery.

TABLE IV-10

EFFECTS OF COMPOSITION OF THE MEDIUM ON

RED CELL RECOVERY OBTAINED BY PROCESS II

% Plasma[a]	% PVP[b]	Concentration of Salt in Additive[c]		
		0.0 M	0.05 M	0.15 M
15	10	90(78)	93(80)	92(60)
15	20	96(88)	96(86)	97(79)
15	30	97(83)	96(57)	97(66)
30	10	94(88)	97(88)	93(65)
30	20	97(88)	97(87)	97(81)
30	30	98(80)	97(64)	97(70)
60	10	95(81)	94(84)	94(71)
60	20	98(90)	95(84)	97(83)

Values are % direct RBC recovery; values in parentheses are % resuspension recoveries in isotonic saline (1:100). All samples consisted of RBCs (1 vol.) + medium (2 vol.). Frozen in uncoated BFF-19110 containers in liquid nitrogen at 200 cpm and thawed in 45° water at 150 cpm.

(a) Vol.% plasma in medium

(b) w/v% K-22 PVP in medium

(c) NaCl conc. (molar) in PVP soln. added - Does not include salt from plasma. (See text).

Albumin at a concentration of 5-7% was observed to effectively substitute for plasma. The complete absence of salt led to reduced red cell recovery. However concentrations of only 0.02 M or above appeared sufficient.

B. PHYSICAL PARAMETERS

Excessively rapid agitation, especially during thawing, reduced red cell direct and resuspension recoveries (Table IV-11). Using mechanical agitation at an amplitude of 2-1/2 inches, an optimum frequency of approximately 200 cycles/minute was observed. Thawing can be carried out at low frequencies of agitation and even manual shaking (approximately 75-100 cycles/minute) is adequate (Table IV-12).

TABLE IV-11

EFFECT OF CONDITIONS OF AGITATION DURING

COOLING AND WARMING ON PROCESS-II

Frequency of Agitation Cycles/Min. (Thawing)	Frequency of Agitation, Cycles/Min. (Cooling)		
	150	225	300
150	94(83)	--	88(89)
175	--	93(87)	--
225	91(86)	--	83(90)
275	--	84(90)	--
300	74(89)	--	70(90)

Values are % Direct RBC Recovery

Values in parentheses are % Resuspension Recovery in Saline

RBC's in equal volume of 50% Plasma-15% K30 PVP-0.1 M NaCl. Frozen (53 ml) in BFF-19110 containers with PVP-MeOH coating, variable agitation, in liquid N_2. Thawed in 45°C water, variable agitation.

TABLE IV-12

EFFECT OF AGITATION CONDITIONS DURING

THAWING ON PROCESS-II

Agitation Frequency Cycles/Min. (Thawing)	Direct RBC Recovery %	% Resuspension Recovery	
		Saline	PVP
Manual (75-100)	96	88	94
Mech. 50-80	96	85	92
Mech. 60-95	95	87	93
Mech. 90-110	96	88	94

RBC's in equal volume of 50% plasma-15% K30 PVP-0.1 M NaCl. Frozen (53 ml) in BFF-19110 containers with PVP-MeOH coating, 200 cpm agitation, in liquid nitrogen. Thawed in 45°C water.

Red cell recoveries decline when thawing is carried out in water at temperatures below 30°C. Equivalent recoveries were obtained when thawings were done in water at 30 to 45°C (Table IV-13).

TABLE IV-13

EFFECT OF TEMPERATURE OF THAWING ON PROCESS-II RECOVERY

Thaw Bath Temperature, °C	Direct % RBC Recovery	% Recovery in Dextran
20	91	96
25	92	96
30	94	97
35	95	97
40	95	98
45	95	97

RBC/Medium = 1:1 ; Medium: 50% plasma - 15% PVP-0.1 M NaCl.

Although more sensitive to such factors as low concentrations of plasma or polyvinylpyrrolidone, samples frozen and thawed in polyethylene containers gave in vitro recoveries equivalent to those obtained in aluminum containers of approximately equal geometries (Table IV-14 and Figure IV-9). Subsequent studies of red cell survival indicated that plastic containers used under comparable conditions to metal containers resulted in reduced viability. Further investigation of plastic containers with thin walls and thawing at higher bath temperatures would appear justified.

TABLE IV-14

COMPARISON OF METAL AND PLASTIC CONTAINERS

FOR PROCESS-II

Container	Direct % RBC Recovery	Resuspension Recovery % Saline	PVP
Metal	98	92	95
Plastic	96	94	97

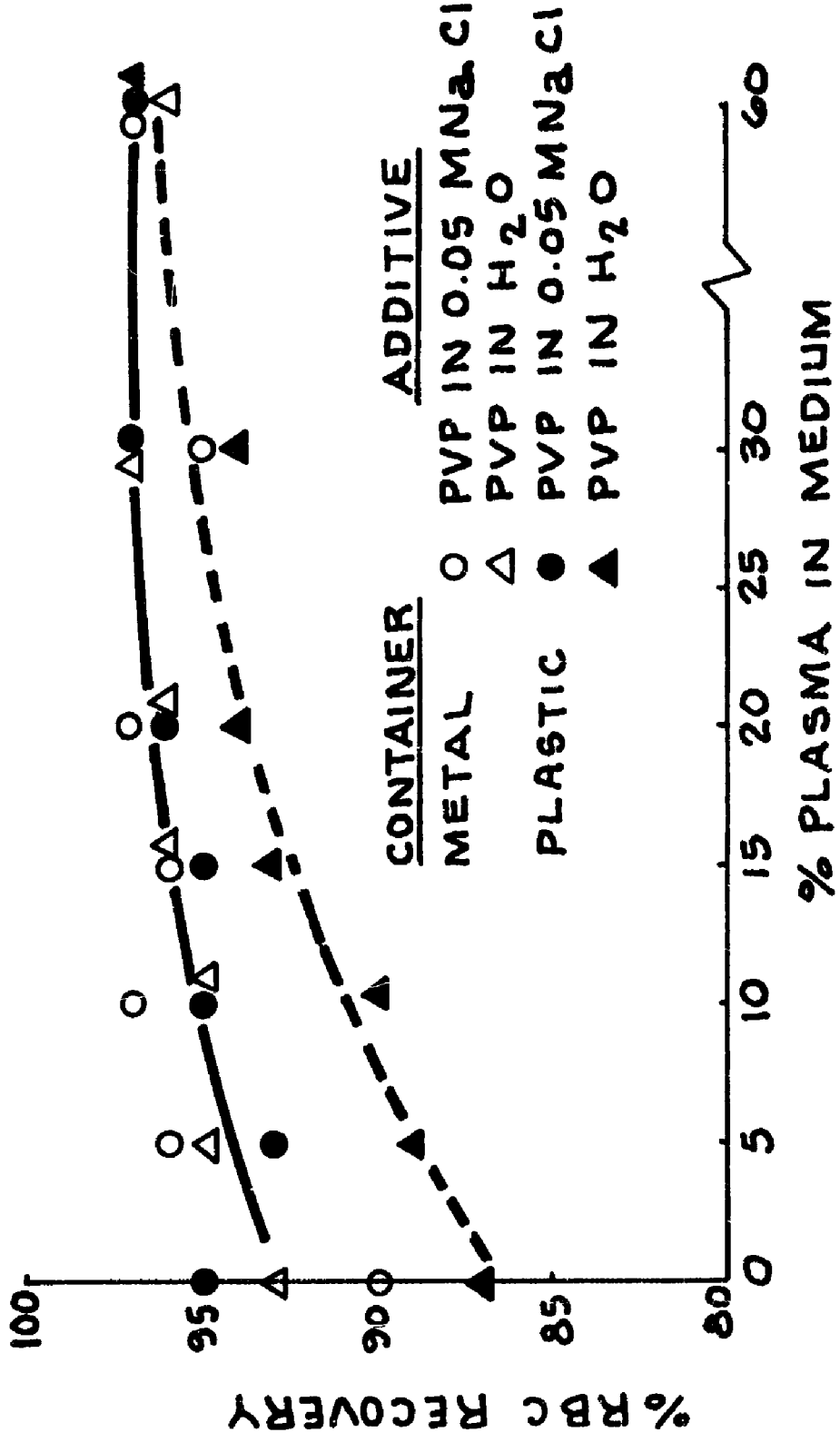
See footnote, Table IV-3.

C. PROPERTIES OF RED CELLS FROZEN BY PROCESS II

Red cells recovered from Process II (RBC/total volume = 1:3) in which the medium consisted of 50% plasma-20% K-22 PVP-0.075 M NaCl were compared to unfrozen control cells with respect to saline osmotic fragility, colloid osmotic fragility, mechanical fragility, and pH fragility. In all cases frozen and control cells were suspended 1:2 in 6% dextran at the time of testing.

No marked differences could be detected in terms of colloid osmotic fragility, mechanical fragility, or pH fragility. However, some 20% of the population of frozen cells were abnormal in terms of saline osmotic fragility. This fraction lysed in buffered saline of any concentration studied (up to 0.6 molar). It is possible that immediate post transfusion loss and perhaps the total loss over the first 24 hours after transfusion is composed of this osmotically unstable population. These cells may be injured in terms of their cation transport system[7].

FIGURE IV - 9


% RBC RECOVERY
100
95
90
85
80
0
5
10
15
20
25
30
60
% PLASMA IN MEDIUM
ADDITIVE
CONTAINER
METAL
PLASTIC
○ PVP IN 0.05 M NaCl
△ PVP IN H_2O
● PVP IN 0.05 M NaCl
▲ PVP IN H_2O

Experiments are in progress to reduce the size of this osmotically labile population by physical and chemical conditions. Several interesting leads involving thawing time and the addition of certain compounds (phospholipids, etc.) in low concentration are under study.

D. PROCESS DEVELOPMENT

From the standpoint of civilian or military medicine under conditions where separation and resuspension of red cells before transfusion would not pose a serious limitation, Process II may afford a useful means of preservation when polymers are used as protective additives.

Figure IV-10 gives the process in flow sheet form. Table IV-15 shows results obtained in processing full units of blood. The high dilution systems involved adding PVP to whole blood without removal of plasma. Volumes up to almost 85% of container capacity (up to 950 ml) have been frozen and thawed with no reduction in recovery of red cells.

2. Process III: Whole Blood-Polymer Systems - No Post-Thaw Processing

In the two processes described previously red cells were partially or completely separated from whole blood and combined with relatively high concentrations of polyvinylpyrrolidone prior to freezing. A third approach to preservation of blood using polymeric additives has been to combine whole blood with a polymer at a total concentration low enough to afford the possibility of subsequent transfusion without separation. Several polymers at concentrations near those employed as plasma expanders were studied under cooling conditions previously found useful for protection by sugars. Results shown in Table IV-16 were obtained. Additional results are described in Section V.

On the basis of animal experiments (Section V) and previous small volume tests [7] a detailed study of polyvinylpyrrolidone in whole blood was undertaken.

A. CHEMICAL PARAMETERS

Polyvinylpyrrolidone (K-30, M.W. 40,000) concentrations of 7% or greater in whole blood and cooling rates slower than those required for sugar additives were found necessary for red cell recoveries approaching 95% (Figures IV-11, IV-12).

The concentration rather than total amount of PVP was shown to be important in protection (Table IV-17).

FIGURE IV - 10

FLOW SHEET FOR PROCESS - II

PRESERVATION OF RED CELLS IN PLASMA-POLYMER MEDIUM

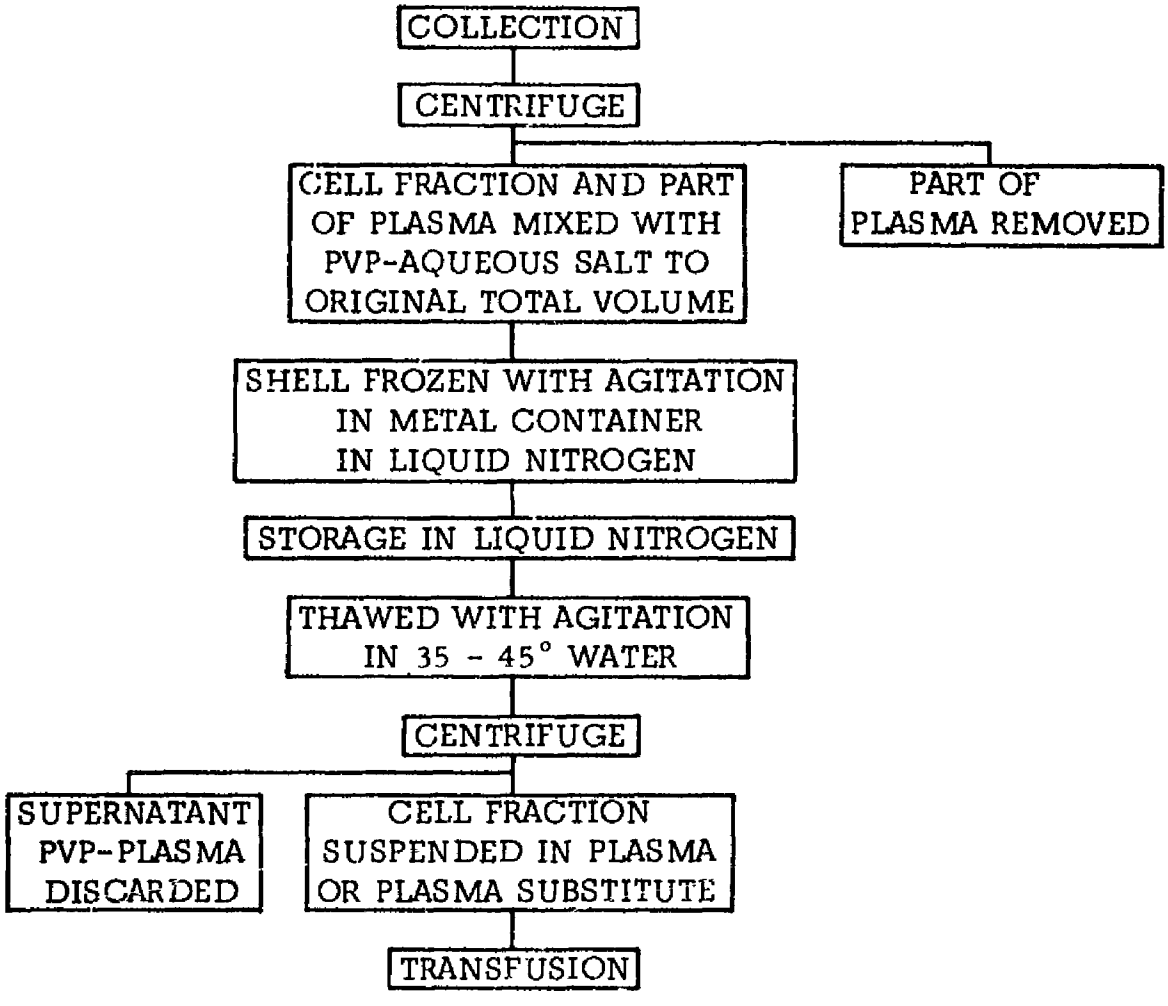


TABLE IV-15

PROCESSING OF PINT VOLUMES OF BLOOD BY PROCESS II

Exp. No	Dilution RBC/Total Vol.	% Direct RBC Recovery	% Resuspension Recovery		
			Dextran[a]	Saline[b]	PVP[b]
1	1:2.6	95	(94) [c]	86	93
2	1:2.6	95	96	--	--
3	1:2.6	96	96	83	86
4	1:2.5	94*	95	87	93
5	1:2.5	97	98	86	91
6	1:4.1	96	98	88	91
7	1:4.7	96	98	--	--

RBCs suspended in 50% Plasma-15% K-30 PVP-0.1 M NaCl in ratios shown. Ratios of 1:2.5-2.6 were systems with 1/2 plasma removed followed by reconstitution to volume with PVP solution. Ratios of 1:4.1-4.7 were systems with PVP solution added directly to whole blood giving volumes of 800-900 ml at time of freezing. All frozen in PVP-MeOH coated pint containers (capacity 1100 ml approximately) at 200 cpm agitation in liquid nitrogen approximately 90 seconds. Thawing in 45° water, 150 cpm, 45-50 seconds.

(a) Resuspended RBCs 1:2

(b) Resuspended RBCs 1:100

(c) Resuspension 1:2 in 5% albumin-saline

* Incompletely thawed in 45 seconds

TABLE IV - 16

RED CELL RECOVERIES OBTAINED IN WHOLE BLOOD

WITH VARIOUS POLYMERIC ADDITIVES

Additive			Volume	Direct %
Type	Conc.%	Container	Frozen, ml.	RBC Recovery
Dextran-10	3	XFC-1/2	279	61
"	6	"	318	57
"	9	"	306	91
Dextran-40	3	XFC-1/2	303	34
"	6	"	314	87
Alginon	2	BFF 1975	45	60 (10)*
"	1	"	45	50 (0)*
PVP K15	3	XFC-1/2	294	36
"	6	"	303	84

Whole blood (4 vols) combined with additive in isotonic saline (1 vol) to give final concentrations shown.

Freezing with mechanical agitation (200 cycles/min, 2-1/2 inch amplitude) in liquid nitrogen with Santocel-glycerol heat transfer coating. Thawing in 45°C water with mechanical agitation.

* Values in parentheses are recoveries after washing thawed cells with buffered saline - 0.4% glucose.

FIGURE IV-11

EFFECT OF POLYVINYLPYRROLIDONE CONCENTRATION IN WHOLE BLOOD

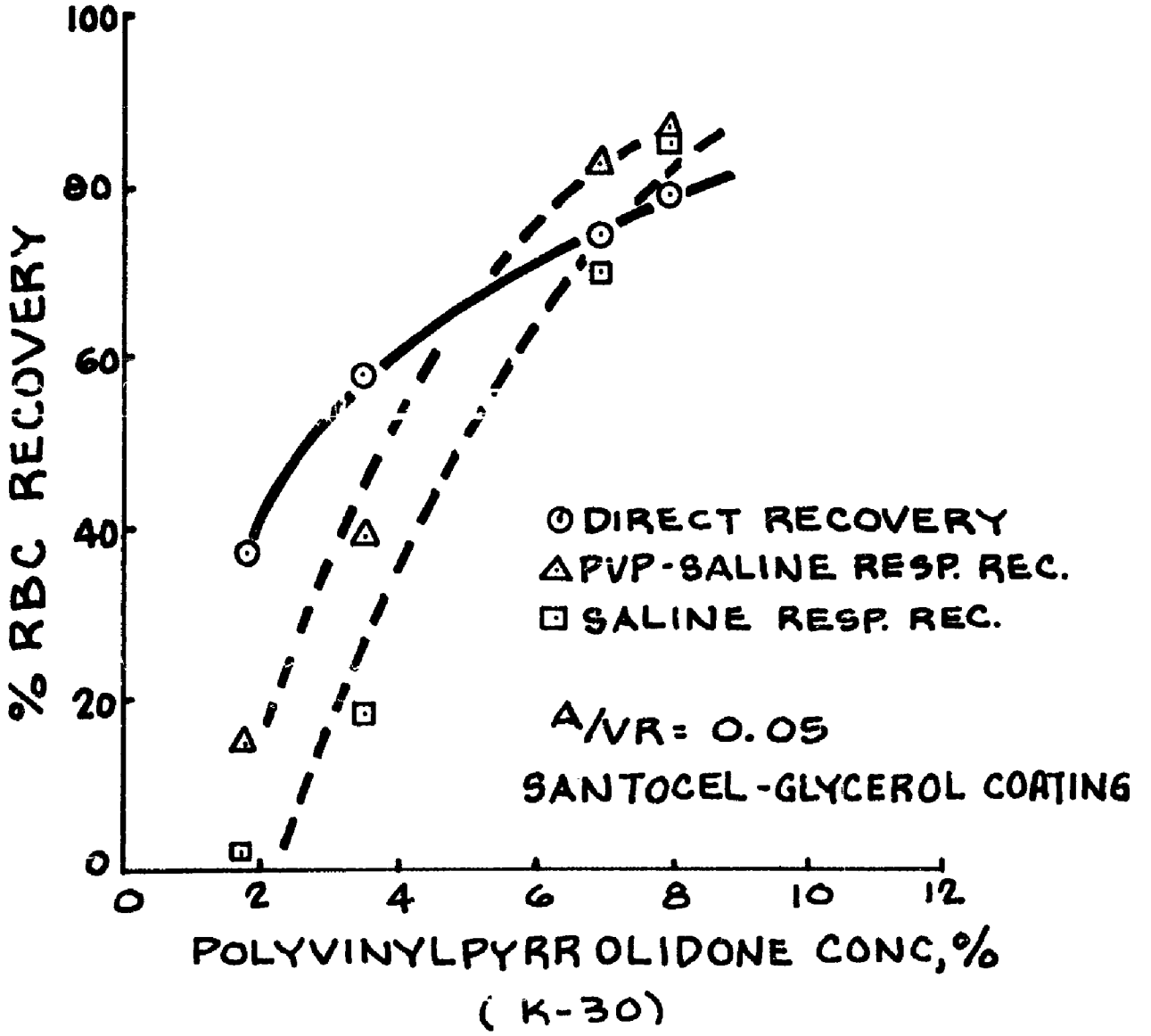


FIGURE IV-12

EFFECT OF POLYVINYLPYRROLIDONE CONCENTRATION WHOLE BLOOD

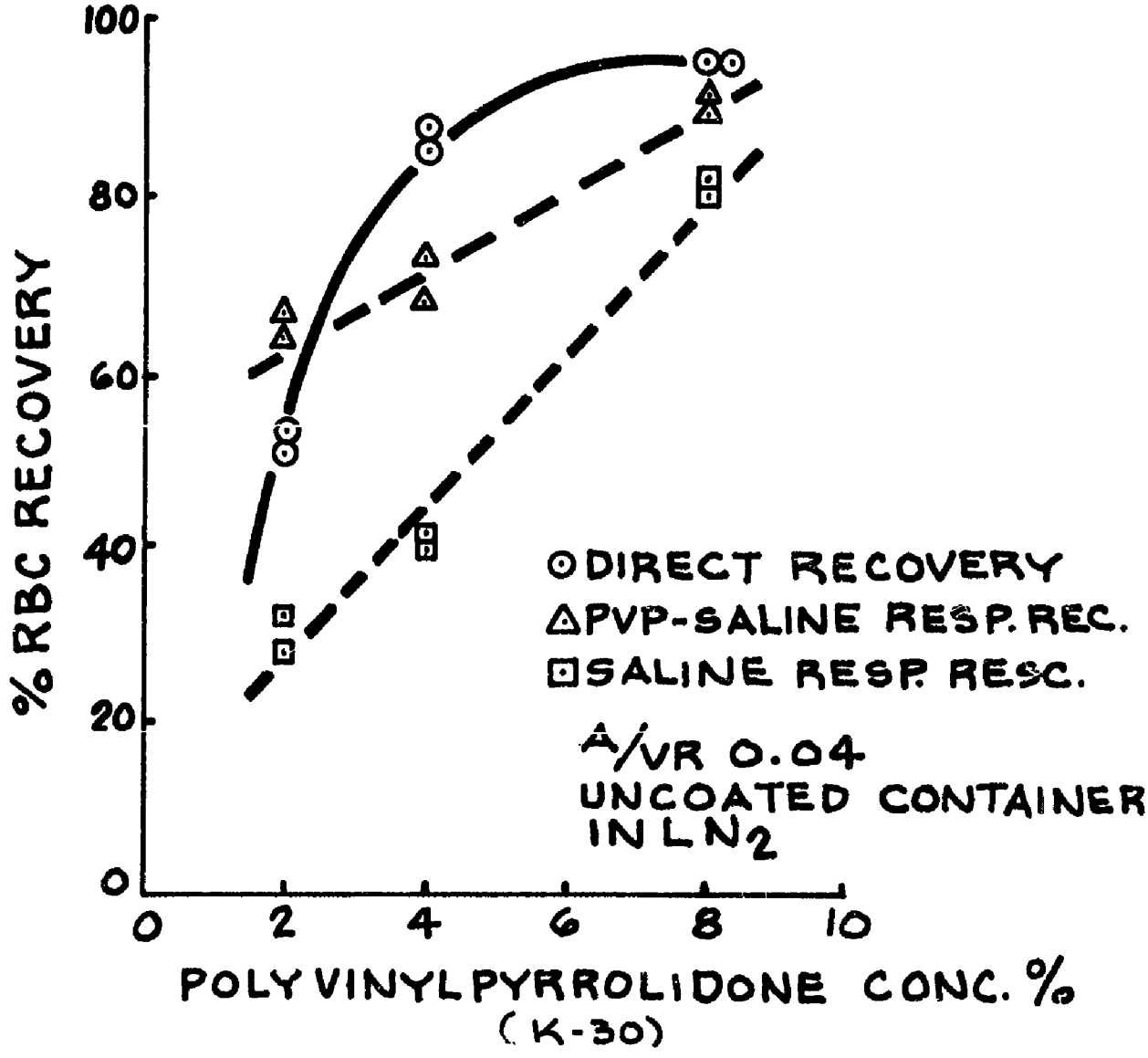


TABLE IV-17

COMPARISON OF K-15 AND K-30 POLYVINYLPYRROLIDONE IN WHOLE BLOOD AT CONSTANT TOTAL CONCENTRATION

Additive	Vol. PVP Soln. per Vol. WB	% PVP in System		% RBC Recovery	% Resusp. Recovery	
		Total	Medium		Saline	PVP
PVP K-30	1	7	8.8	94	78	89
PVP K-30	0.75	7	9.1	94	76	87
PVP K-30	0.5	7	9.5	94	81	88
PVP K-30	0.25	7	10.3	94	81	89
PVP K-15	1	7	8.8	88	53	76
PVP K-15	0.75	7	9.1	87	57	77
PVP K-15	0.5	7	9.5	86	62	80
PVP K-15	0.25	7	10.3	87	58	76

Whole blood combined with PVP in isotonic saline to give total concentrations of 7% in the mixture. Volumes of 53 ml frozen in BFF 19110 containers in liquid nitrogen using PVP-Methanol coatings and mechanical agitation. Thawing with agitation at 45°C.

Direct red cell recoveries and resuspension recoveries increased continuously with increase in average molecular weight of the PVP employed for protection (Figure IV-13). Recoveries as high as 95%, however, were obtained with PVP K-22 (M. W. 25,000) under suitable cooling conditions (Table IV-18).

A slight but reproducible beneficial effect on resuspension recovery was obtained by adding low concentrations of glucose to PVP K-22 (M. W. 25,000). No significant effect was observed on direct red cell recovery (Table IV-19). A similar slight effect was observed with K-15 and K-30 PVP's (Table IV-20).

B. PHYSICAL PARAMETERS

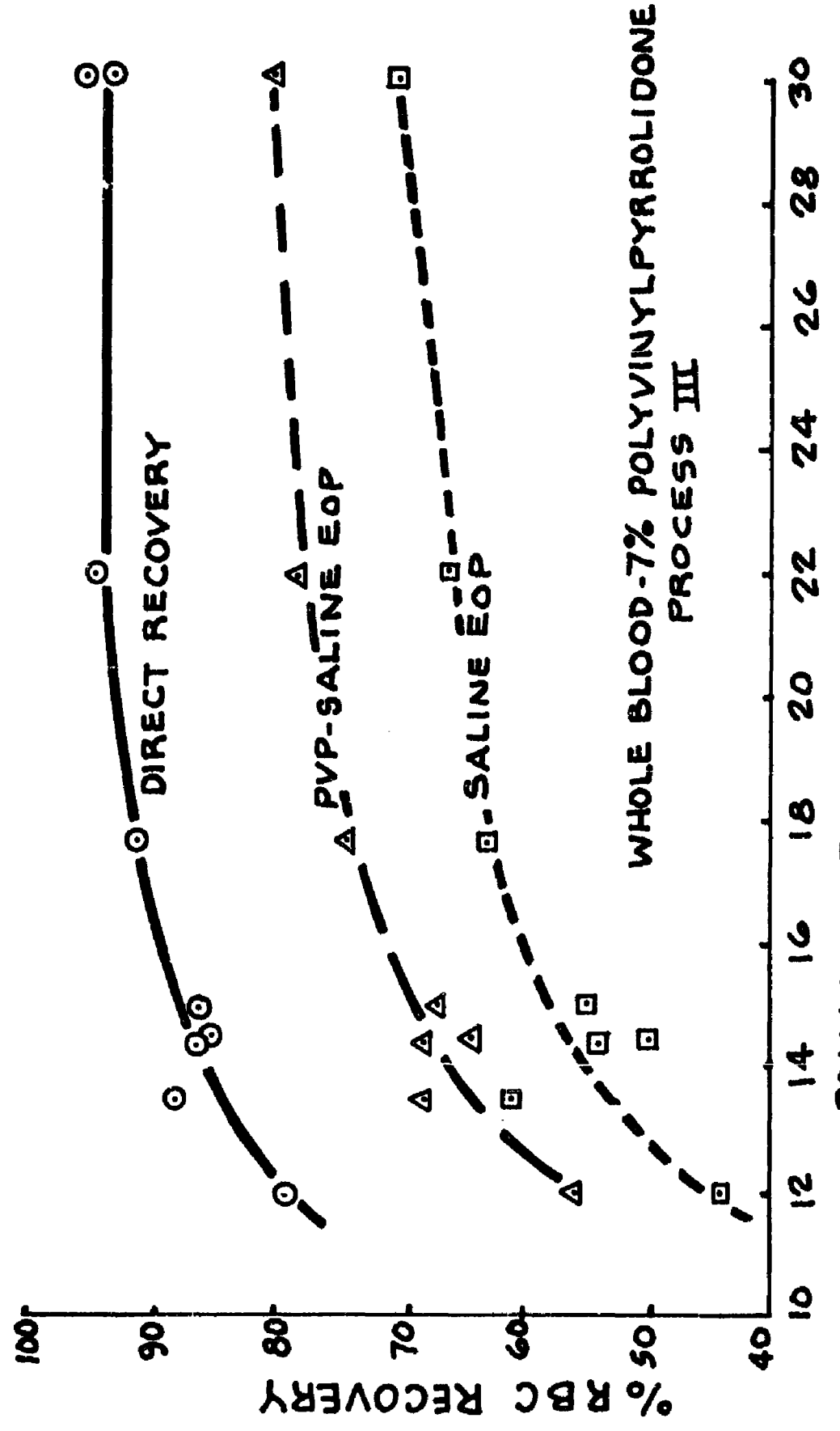
As shown in Table IV-21 slower rates of cooling are required for polymer protection than for protection by sugars. Comparison of cooling conditions for protection by PVP's K-15 and K-30 at 5% and 7% concentrations respectively are shown in Table IV-22 and Figure IV-14. Optimum conditions at relatively low A/VR ratios existed in each case. Maximum recoveries were obtained using PVP-Methanol coating on the containers rather than Santocel-Glycerol.

Variation in the thickness of the PVP coating produced only slight change in recoveries. Thickness of coating increased as viscosity was increased by increasing PVP concentration (Figure IV-15 and Table IV-23).

Direct and resuspension recoveries could be improved by increasing agitation frequency during freezing from 50 to 200 cycles/minute (Table IV-24). Studies with rabbit blood (Section V) indicated that agitation above about 200 cpm during thawing decreased recovery and survival.

FIGURE IV-13

RED CELL RECOVERY AS A FUNCTION OF K-VALUE FOR WHOLE BLOOD - 7% POLYVINYLPYRROLIDONE



Frozen in BFF-19110 containers with PVP-Methanol heat transfer coating and mechanical agitation in liquid N_2. Thawed with agitation in 45° water.

TABLE IV - 18

PROCESS III PVP K-22

SMALL VOLUMES

Volume Frozen (cc)	[PVP] in Blood Mixture (%)	Normal Recovery (%)	Thawing Conditions 45°C 160 cpm Time (sec)	Saline EOP (%)
64.8	8	95.6	35	77.6
62.5	7	93.2	33-1/2	67.8
61.4	6.5	93.0	32	65.6
60.4	6	92.6	32	62.6
58.3	5	90.1	32	55.0
50	8	95.6	29	78.3
50	7	95.2	29	73.9
50	6.5	94.1	29	68.8
50	6	93.3	30	67.0
50	5	89.8	32	61.3

Blood: Drawn into ACD-B by Red Cross. For first 5 samples 50 cc of blood were added directly to additives in containers. For last 5 samples blood mixtures were prepared in flasks and appropriate quantities transferred to containers.

Additive: 35% PVP (K-22) in .056M Saline.

Container: Flat rectangular 110 cc aluminum container, (BFF 19-110) uncoated.

Saline EOP: 1 volume of the thawed blood preparation is added to 99 volumes of saline and allowed to stand 1/2 hour prior to analysis.

TABLE IV - 19

PROCESS III PVP K-22

GLUCOSE EFFECT

Additive Composition			Normal Recovery	Saline EOP
PVP (%)	NaCl (M)	Glucose (%)	(%)	(%)
40	0	0	96.4	83.8
40	0	2	95.9	82.9
40	0	4	95.9	82.1
40	0	6	96.5	85.7
40	.03	0	96.0	80.1
40	.03	2	96.1	82.1
40	.03	4	96.9	83.2
40	.03	6	97.4	86.4

Blood: Drawn into ACD-B by Red Cross. Approximately 42.5 cc of blood were added to 9 cc of additive in containers.

Container: BFF 19-110, uncoated

Thaw Conditions: 160 cpm agitation in 45°C water bath.

Saline EOP: 1 volume of thawed blood is diluted 100 fold with physiological saline and allowed to stand 1/2 hour at room temperature prior to analysis.

TABLE IV-20

EFFECT OF GLUCOSE ON PROTECTION BY

POLYVINYLPYRROLIDONE IN WHOLE BLOOD

Composition of System	% Direct RBC Recovery	% RBC Resuspension Recovery[a]
WB-Glucose 0.25M	52	15
WB-PVP K-15, 3.5%	60	38
WB-PVP K-30, 3.5%	75	17
WB-PVP K-30, 3.5%-Glucose 0.25M*	72	23
WB-PVP K-15, 3.5%-Glucose 0.25M*	70	40
WB-PVP K-30, 3.5%-Glucose 0.25M**	71	28
WB-PVP K-15, 3.5%-Glucose 0.25M**	77	34

Whole blood-citrate anticoagulant (4 vol.) combined with additive (1 vol.) in isotonic saline to final concentrations shown for mixture. Frozen in BFF 1975 containers with PVP-Methanol coating with mechanical agitation (200 cycles/min. 2-1/2 inch amplitude) in liquid nitrogen. Thawed in 45°C water with mechanical agitation (same as freeze).

(a) Recovered RBCs resuspended in 10 volumes buffered saline containing 0.4% glucose.

* Glucose and PVP added simultaenously; ** glucose added and equilibrated with blood 1 hour before PVP addition.

TABLE IV-21

EFFECT OF VOLUME AND HEAT TRANSFER COATING ON PROTECTION AFFORDED BY POLYVINYLPYRROLIDONE (K-15) IN WHOLE BLOOD

Volume Frozen as % of Container Capacity	Direct % RBC Recovery	
	PVP-Methanol	Santocel-Glycerol
74	--	74±5
66	--	72±3
60	84±2	71±1
53	85±1	75±1
46	88±1	74±1
40	91±1	--
34	92±1	--

Whole blood (4 vol.) combined with K-15 polyvinylpyrrolidone in isotonic saline (1 vol.) to give final concentration of 7% in the mixture. Frozen in BFF 1975 containers in liquid nitrogen and thawed in 45°C water. Mechanical agitation on cooling and warming at 200 cycles/minute, 2-1/2 inch amplitude.

TABLE IV-22

RED CELL RECOVERY AND RESUSPENSION STABILITY OBTAINED WITH WHOLE BLOOD - POLYVINYLPYRROLIDONE (K 15) SYSTEMS UNDER VARIOUS COOLING CONDITIONS

A/VR	Direct % RBC Recovery	Resuspension Recovery % Saline	PVP
0.02	82	54	70
0.04	86	64	78
0.05	85	44	56
0.06	64	47	46
0.07	60	48	54
0.08	56	52	62

Whole blood (4 vols) combined with K 15 PVP 25% in isotonic saline (1 vol). Volume and Heat Transfer coating varied. Thawed at 45° C. Container: BFF-19110s.

FIGURE IV-14

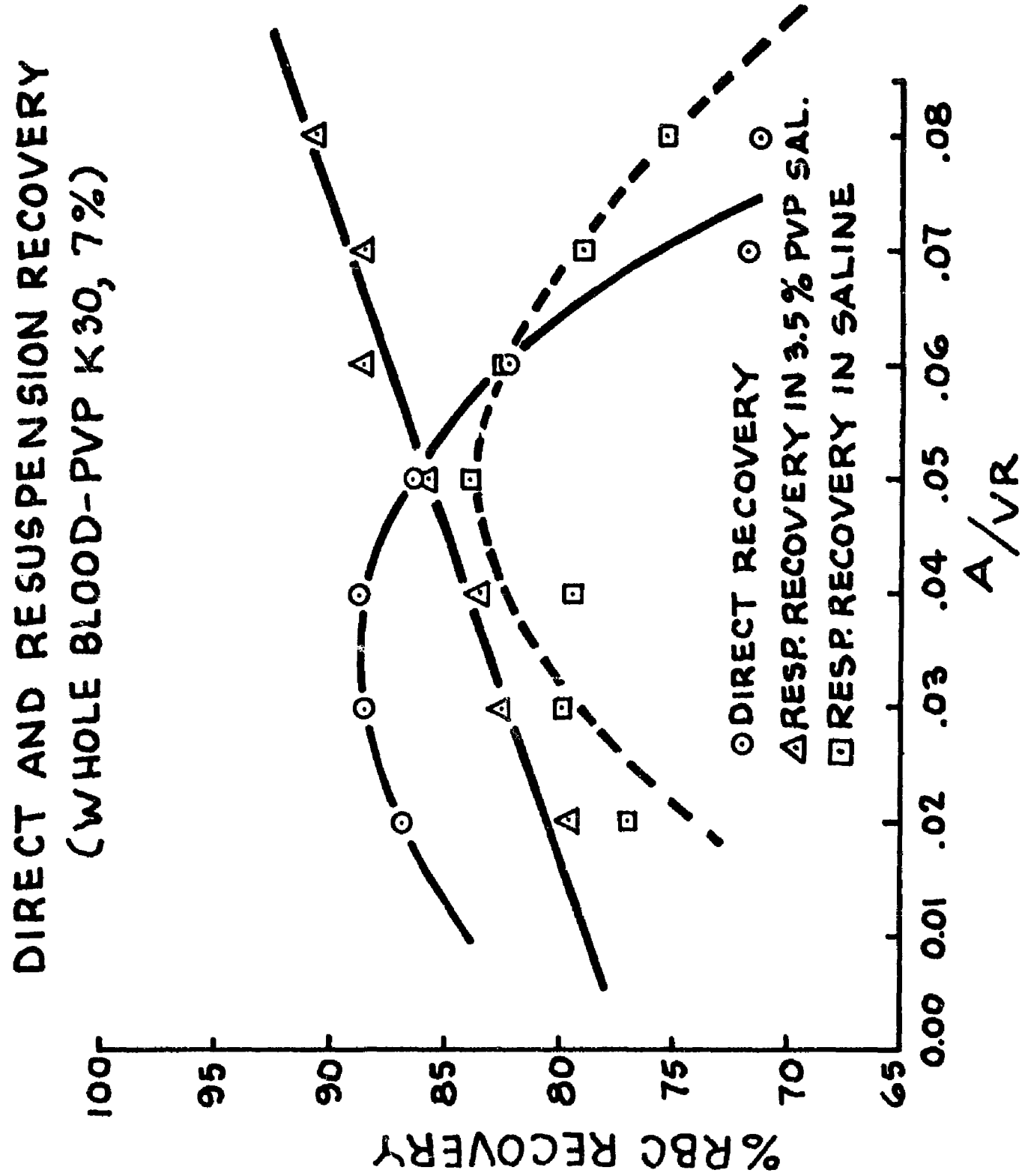

DIRECT AND RESUSPENSION RECOVERY
(WHOLE BLOOD-PVP K30, 7%)
⊙DIRECT RECOVERY
△RESP. RECOVERY IN 3.5% PVP SAL.
⊡RESP. RECOVERY IN SALINE
% RBC RECOVERY
100
95
90
85
80
75
70
65
0.00
0.01
.02
.03
.04
.05
.06
.07
.08
A/VR

FIGURE IV-15

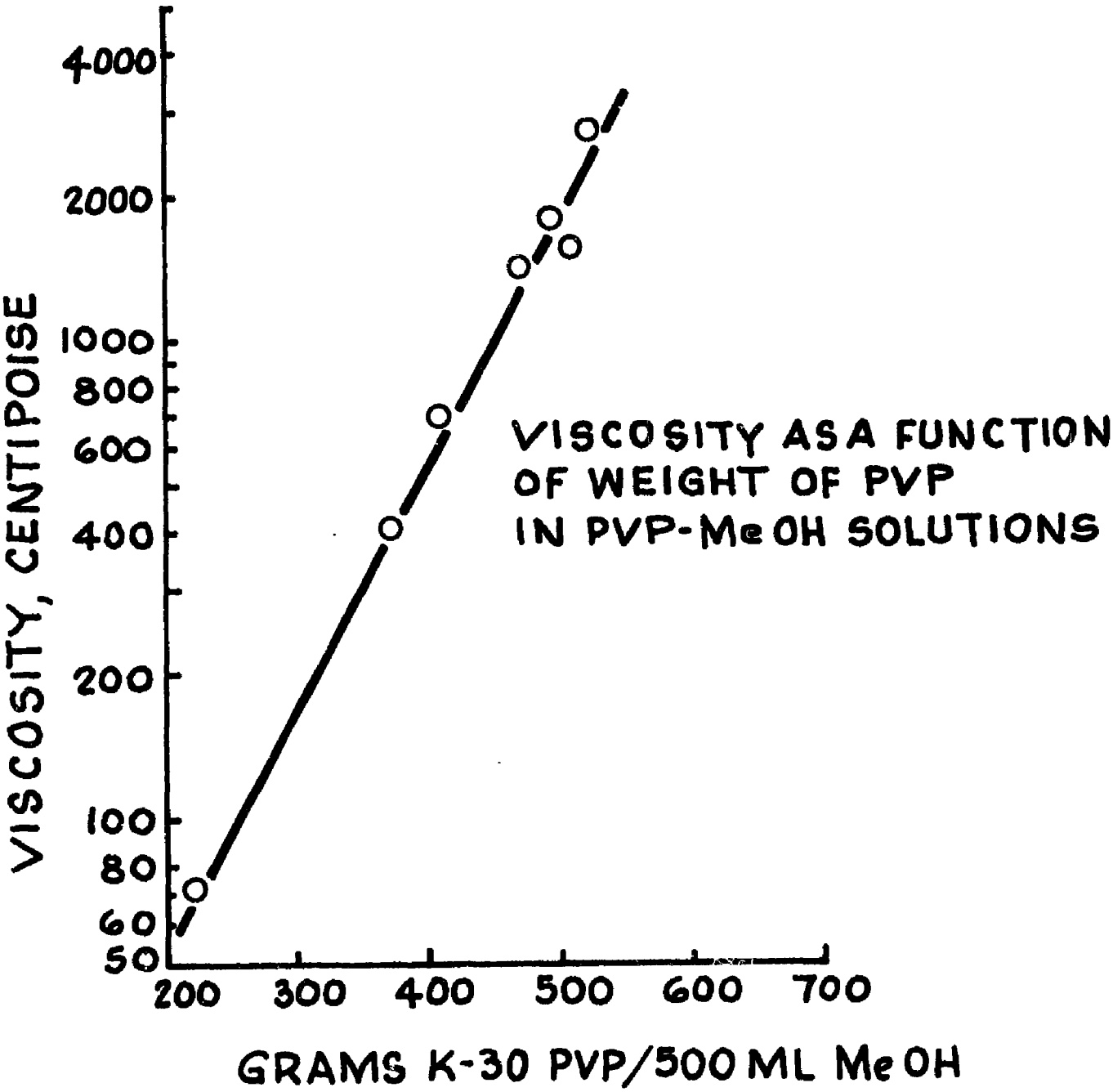

VISCOSITY, CENTIPOISE
4000
2000
1000
800
600
400
200
100
80
60
50
200
300
400
500
600
700
GRAMS K-30 PVP/500 ML MeOH
VISCOSITY AS A FUNCTION
OF WEIGHT OF PVP
IN PVP-MeOH SOLUTIONS

TABLE IV-23

EFFECT OF VARYING THE PROPERTIES

OF THE PVP HEAT TRANSFER COATING

Viscosity PVP Coating*	% RBC Recovery	% Resuspension Recovery	
		Saline	PVP
73.5 cp.	94	85	93
411 cp.	95	85	94
703 cp.	95	86	94
1595 cp.	96	89	95

*In Centipoise (PVP in Methanol)

Whole blood combined 4:5 with 35% K-30 PVP in 0.15 M NaCl (final conc. PVP in mixture 7%). Fifty-three ml frozen in BFF-19110s in liquid nitrogen with agitation at 200 cpm. Thawing in 45°C water at 200 cpm.

TABLE IV-24

EFFECT OF CONDITIONS OF AGITATION FOR WHOLE

BLOOD - PVP SYSTEM

Frequency of Agitation CPM		% RBC	% Resuspension Recovery	
Cooling	Warming	Recovery	Saline	PVP
50	50	94	80	90
50	200	93	80	89
200	50	95	84	93
200	150	96	86	94
200	200	95	85	94

Whole blood combined 4:5 with 35% K-30 PVP in 0.15 M NaCl. Fifty-three ml in BFF-19110 containers coated with K-30 PVP in methanol (411 cp), frozen in liquid nitrogen. Thawed in 45°C water.

C. PROCESS DEVELOPMENT

Process III is shown in flow diagram in Figure IV-16. Half-pints and full pints of blood have been processed with red cell recoveries of 96-98% when PVP was present at 7% concentration. PVP K-22 appears to be somewhat less protective than K-30 PVP, but conditions of cooling can be varied to obtain recoveries of 97% using K-22.

Results of large volume experiments are shown in Tables IV-25 through IV-27, and Figure IV-17. Post-thaw stability of blood frozen and thawed by Process III is high when storage is carried out at 4°C. Only 1% loss occurred over a period of 168 hours (Table IV-28).

4. Process IV - Reduced Volume Systems

An approach providing a process requiring a minimum of post-thaw handling, yet allowing the use of higher concentrations of additive during freezing and thawing than would be acceptable for direct transfusion, was sought in a reduced volume system. If red cells are frozen in a small volume of concentrated medium, post-thaw dilution, without separation of the cells, could suffice simultaneously to reduce the additive concentration to physiologic levels and the hematocrit to a normal range. Systems employing this principle have been studied, using polyvinylpyrrolidone or human serum albumin.

A. PROTECTION BASED ON POLYVINYLPYRROLIDONE

When whole blood is collected into, or combined with, 30% K-30 polyvinylpyrrolidone in 0.05 M NaCl equal in volume to the volume of plasma, freezing can be carried out with recovery of 97-98% of the red cells. If, prior to freezing, the blood-PVP mixture is centrifuged and plasma-PVP removed leaving 1/4 volume relative to the red cell volume, freezing and thawing can be carried out with recovery of 95-96% of the red cells. Dilution of 5 volumes of the concentrated (reduced volume) mixture with isotonic saline to 8 volumes reduces the hematocrit to about 45% and the PVP concentration to about 3-3/4% (excluding trapped PVP in the red cell mass). After dilution the recovery of red cells is 90-93%.

1. Chemical Parameters

Under reduced volume conditions the optimum concentration of PVP K-30 was observed to be about 15-17.5% (Table IV-29). PVP's of K-values of 22 or greater (M.W.'s 25,000-40,000) were essentially equivalent (Figure IV-18).

FIGURE IV - 16

FLOW SHEET FOR PROCESS - III

WHOLE BLOOD - POLYMER SYSTEMS

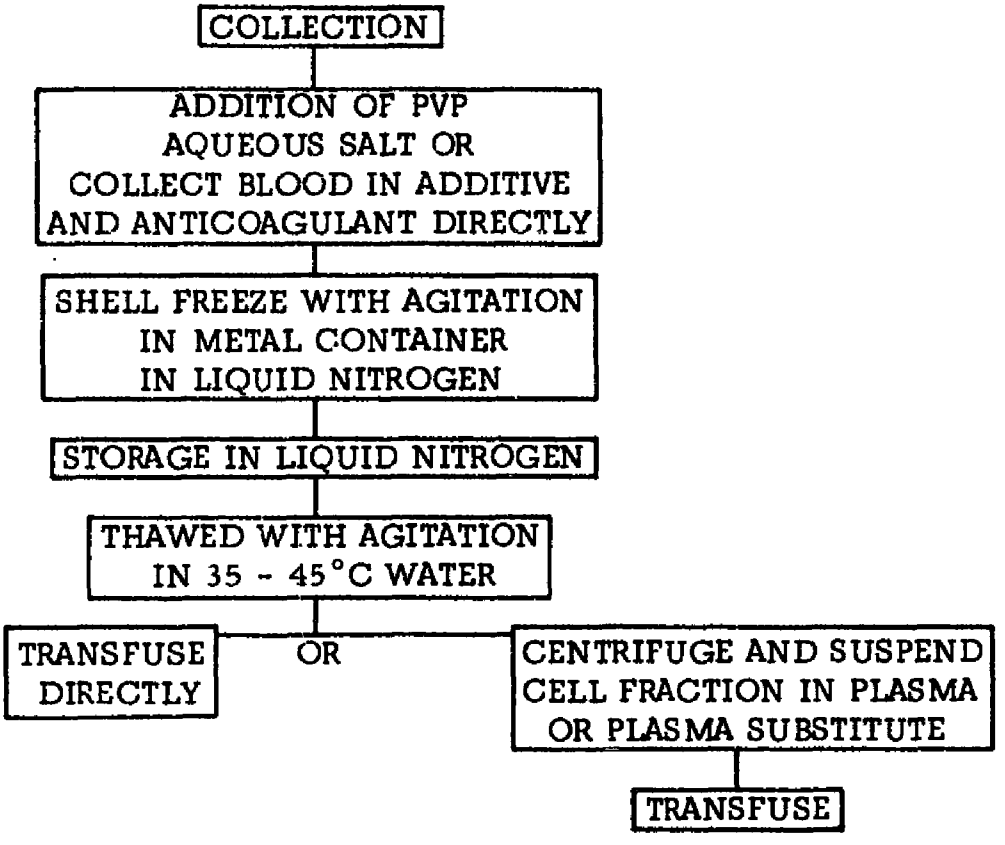


TABLE IV -25

Process III PVP K-22

Half Pints

Volume 35% PVP Solution per 250 cc Blood (cc)	[PVP] in Blood Mixture (%)	[NaCl] in PVP Solution (M)	Direct RBC Recovery (%)	Thawing Conditions 45°C	Saline EOP (%)
62.5	7	.056	96.5	160 cpm 53 sec.	81.6
62.5	7	.056	92.6	150 cpm 60 sec.	67.0
62.5	7	.056	93.8	150 cpm 60 sec. 15 min. stagnant	72.9
62.5	7	0	95.4	160 cpm 53 sec.	77.6
57.0	6.5	.056	96.1	160 cpm 50 sec.	77.1
57.0	6.5	0	94.6	160 cpm 50 sec.	72.0

Blood: Drawn into ACD-B by Red Cross. Blood and additive mixed directly in containers.

Additive: 35% K-22 PVP solution containing NaCl as shown.

Container: Corrugated 1/2-pint aluminum containers (FC-1/2), uncoated.

Saline EOP: 1 volume of the thawed blood preparation is added to 99 volumes of saline and allowed to stand 1/2 hour prior to analysis.

TABLE IV - 26

Process III Plasdone-C

Data Replication for Clinical Testing

InVitro Age of Blood (days)	Volume Frozen (≈ pints)	Direct RBC Recovery (%)	Saline EOP (%)
3	1/2	97	90
3	"	97	89
0	"	96	90
0	"	97	91
0	"	96	88
0	"	97	89
0[a]	"	97	91
0[a]	"	97	91
1	1	97	89
1	1	96	89
0	1/2	97	89
0	"	97	88
0	"	96	88
0[a]	1	97	88
0[a]	1	96	89
0[a]	1/2	98	92
0[a]	"	98	92
0[a]	"	97	89
0[a]	1	97	89
0[a]	"	96	84
0[a]	"	96	87
0[a]	"	97	90
0[a]	"	97	90
0[a]	"	97	90

[a] Blood collected directly into an additive-anticoagulant mixture.

Additive: 35% Plasdone-C in 0.05M NaCl solution. Overall PVP concentration in blood preparation is 7%.
Anticoagulant: ACD-B
Container: Corrugated aluminum containers
Coating: PVP-methanol mixture of approximately 500 cp.
Freezing: 200 cpm agitation in liquid nitrogen
Thawing: 160 cpm agitation in 45°C water bath
Saline EOP: 1 volume of the thawed blood preparation is added to 99 volumes of saline and allowed to stand 1/2 hour prior to analysis.

Data include analyses of samples clinically evaluated.

TABLE IV - 27

Process III PVP K-22

Heat Transfer Coatings

Coating (In order of increased cooling rates)	Direct RBC Recovery (%)	Saline EOP (%)
none	95	75
1 coat varnish	94	74
PVP-methanol (200-250 cp)	97	84
3 coats varnish	97	83
PVP-methanol (500 cp)	97	82
PVP-PVP powder	91	81

Blood Preparation: 250 cc of blood drawn into ACD-B mixed with 63 cc of 35% PVP K-22 in a 1/2-pint aluminum (FC-1/2) container.

Saline EOP: 1 volume of the thawed blood preparation is added to 99 volumes of saline and allowed to stand 1/2 hour prior to analysis.

FIGURE IV-17

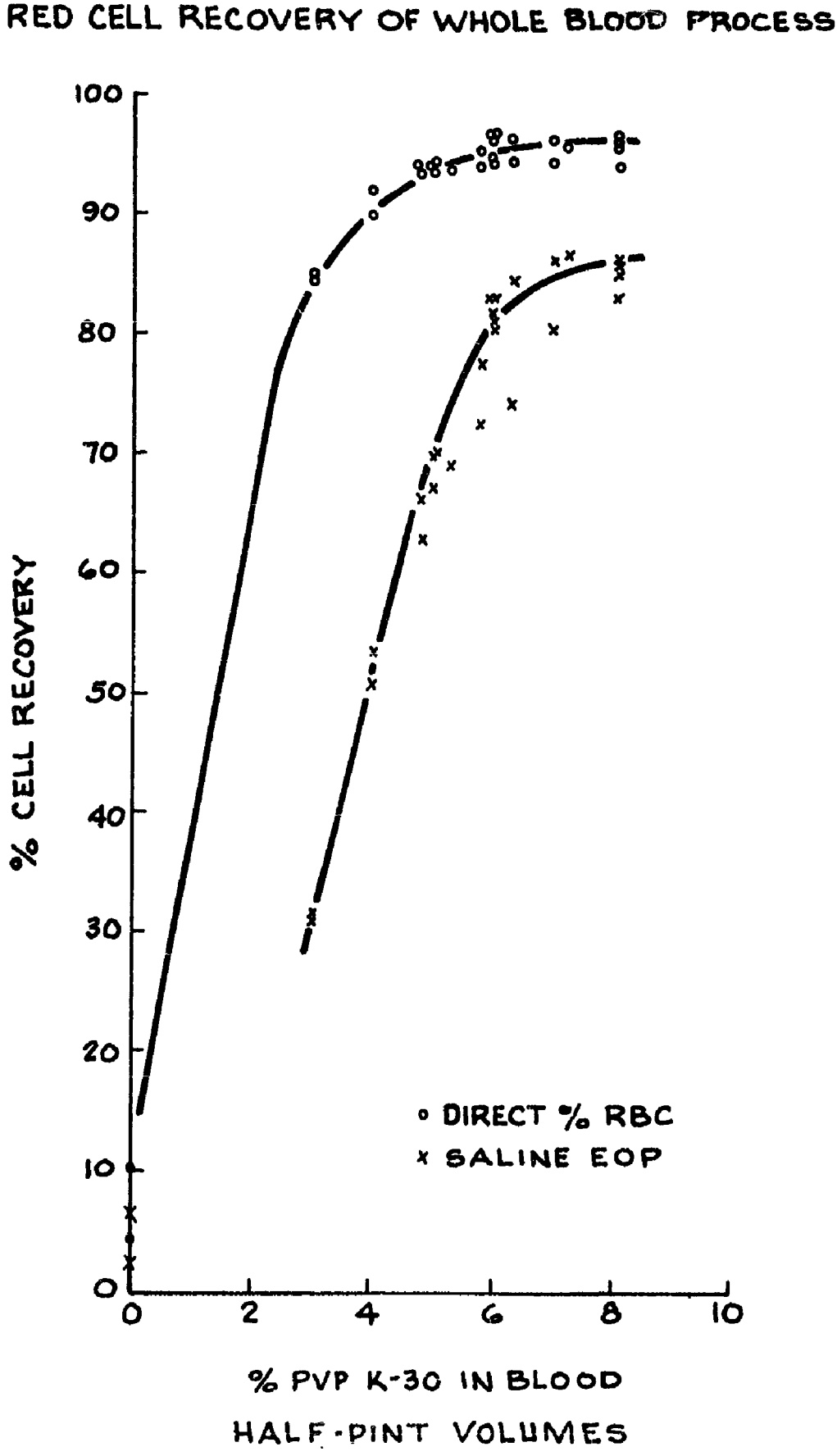

RED CELL RECOVERY OF WHOLE BLOOD PROCESS
% CELL RECOVERY
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
o DIRECT % RBC
x SALINE EOP
% PVP K-30 IN BLOOD
HALF-PINT VOLUMES

TABLE IV - 28

Post Thaw Stability of Process III Blood

Time After Thawing (Hrs)	% Intact Cells			
	Sample A		Sample B	
	Direct RBC Recovery	Saline EOP	Direct RBC Recovery	Saline EOP
1/2	97.4	89.2	97.4	89.8
24	96.8	88.8	96.8	89.5
48	96.5	87.6	96.4	88.9
72	96.6	88.1	96.3	88.6
96	96.5	87.2	96.4	87.3
144	96.4	86.5	96.2	88.1
168	96.5	85.4	96.4	88.2

Sample A: Blood drawn into 72 cc ACD-A, 80 cc 50% Plasdone-C in H_2O and 5 gm glucose to a volume of 550 cc.

Sample B: Blood drawn into 72 cc ACD-A, 80 cc 50% Plasdone-C in H_2O to a volume of 560 cc.

Entire volume frozen in corrugated aluminum containers coated with a PVP-methanol coating, stored in vapor space of a liquid N_2 refrigerator and thawed in a 45°C water bath with 160 cpm agitation.

Thawed blood kept at room temperature for 1-2 hours with subsequent storage at 4°C.

Saline EOP: 1 volume of the thawed blood preparation is added to 99 volumes of saline and allowed to stand 1/2 hour prior to analysis.

FIGURE IV-18

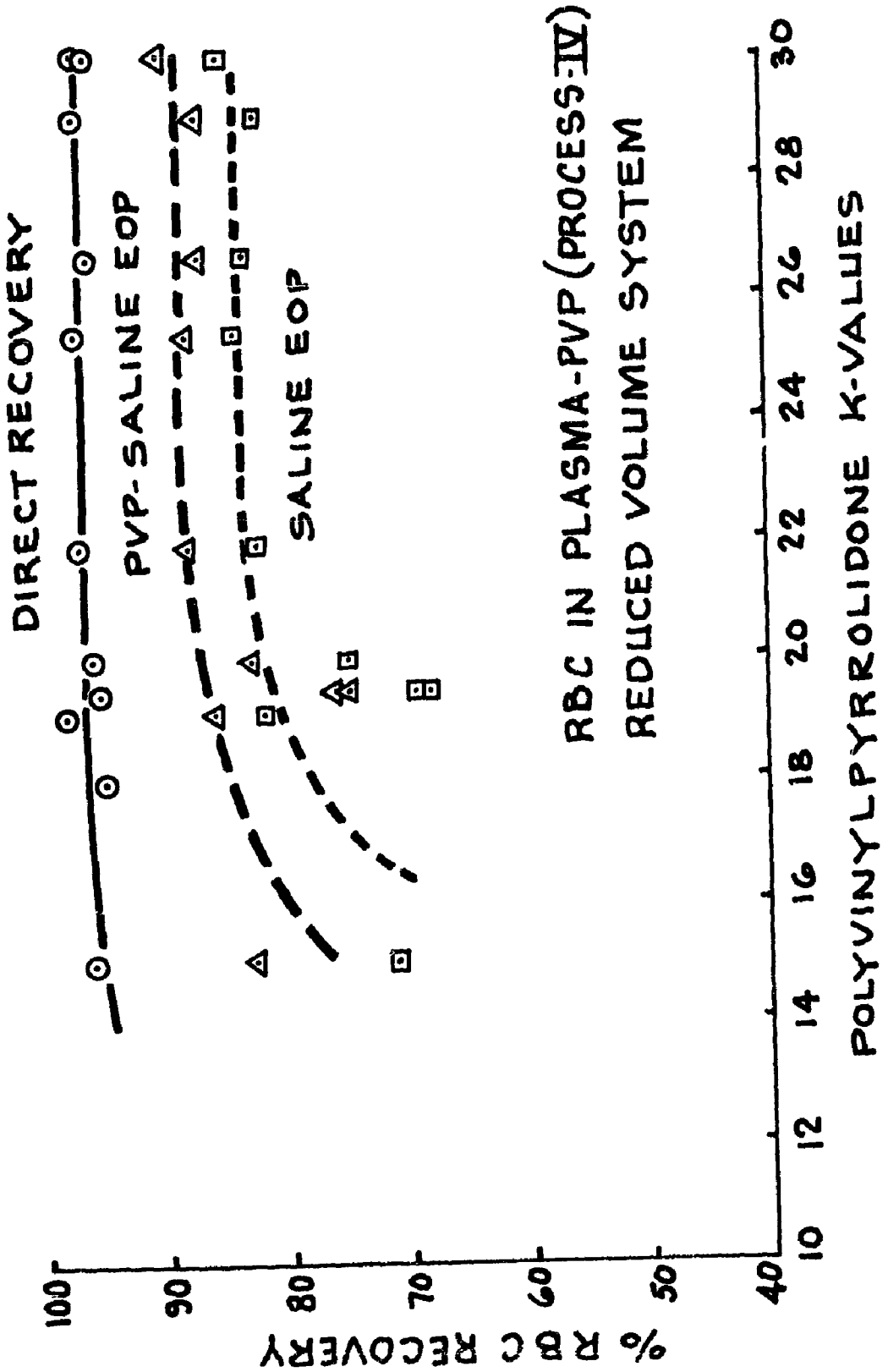

RBC IN PLASMA-PVP (PROCESS-IV)
REDUCED VOLUME SYSTEM
DIRECT RECOVERY
PVP-SALINE EOP
SALINE EOP
% RBC RECOVERY
100
90
80
70
60
50
40
10
12
14
16
18
20
22
24
26
28
30
POLYVINYLPYRROLIDONE K-VALUES

TABLE IV-29

EFFECT OF POLYVINYLPYRROLIDONE CONCENTRATION

IN REDUCED VOLUME SYSTEM

PVP Conc. % in Medium	Direct % RBC Recovery	% Resuspension Recovery	
		Saline	PVP
12.5	95	81	90
15.0	96±1	87±0	93±0
17.5	96±1	87±2	94±1
20.0	94	84	93

RBC's suspended in 1/4 volume of 50% plasma-PVP-0.1 M NaCl. Frozen in BFF-19110 containers with PVP-MeOH coating, 200 cpm agitation, in liquid N_2. Thawed at 150 cpm in 45°C water.

The degree of post-thaw dilution markedly affects red cell recovery as shown in Table IV-30. Variation in recovery after dilution was observed unless dilution was carefully controlled.

TABLE IV-30

EFFECT OF POST THAW DILUTION ON

RECOVERY OBTAINED IN REDUCED VOLUME

POLYVINYLPYRROLIDONE SYSTEM (PROC. IV)

Dilution Factor *	Final Conc. PVP in Medium %(W/V)	% RBC Recovery
1.0	20.7	97
1.2	10.3	95
1.6	5.2	94
2.0	4.1	90
4	1.4	82
10	0.4	77

RBC's processed by Process-IV and diluted with isotonic saline after thawing. K30 PVP used at 20.7% conc. in medium.

* Ratio of thawed blood - PVP mixture to total diluted volume.

Post-thaw stability is shown in Table IV-31. Two to 4% loss per day occurred at 4°C.

TABLE IV-31

POST THAW STABILITY OF RED CELLS IN

REDUCED VOLUME SYSTEM

	% RBC Recovery				
	Time of Storage (Hours)				
Conditions	0	24	48	72	96
Stored Undiluted at 4°C	96	96	--	--	--
Stored Diluted 5:8 at 4°C	93	92	--	--	--
Stored Undiluted at 4°C, but Diluted before Analysis	93	89	87	--	84

Samples consisted of RBC's frozen in 1/4 volume 50% Plasma-15% PVP-0.1 M NaCl and stored as shown.

2. Physical Parameters

Optimum cooling conditions were obtained using uncoated and PVP-methanol coated metal containers. Slower or more rapid heat transfer conditions resulted in markedly reduced recoveries (Figure IV-19).

Metal containers gave superior recoveries when compared to polyethylene containers (Table IV-32).

FIGURE IV-19

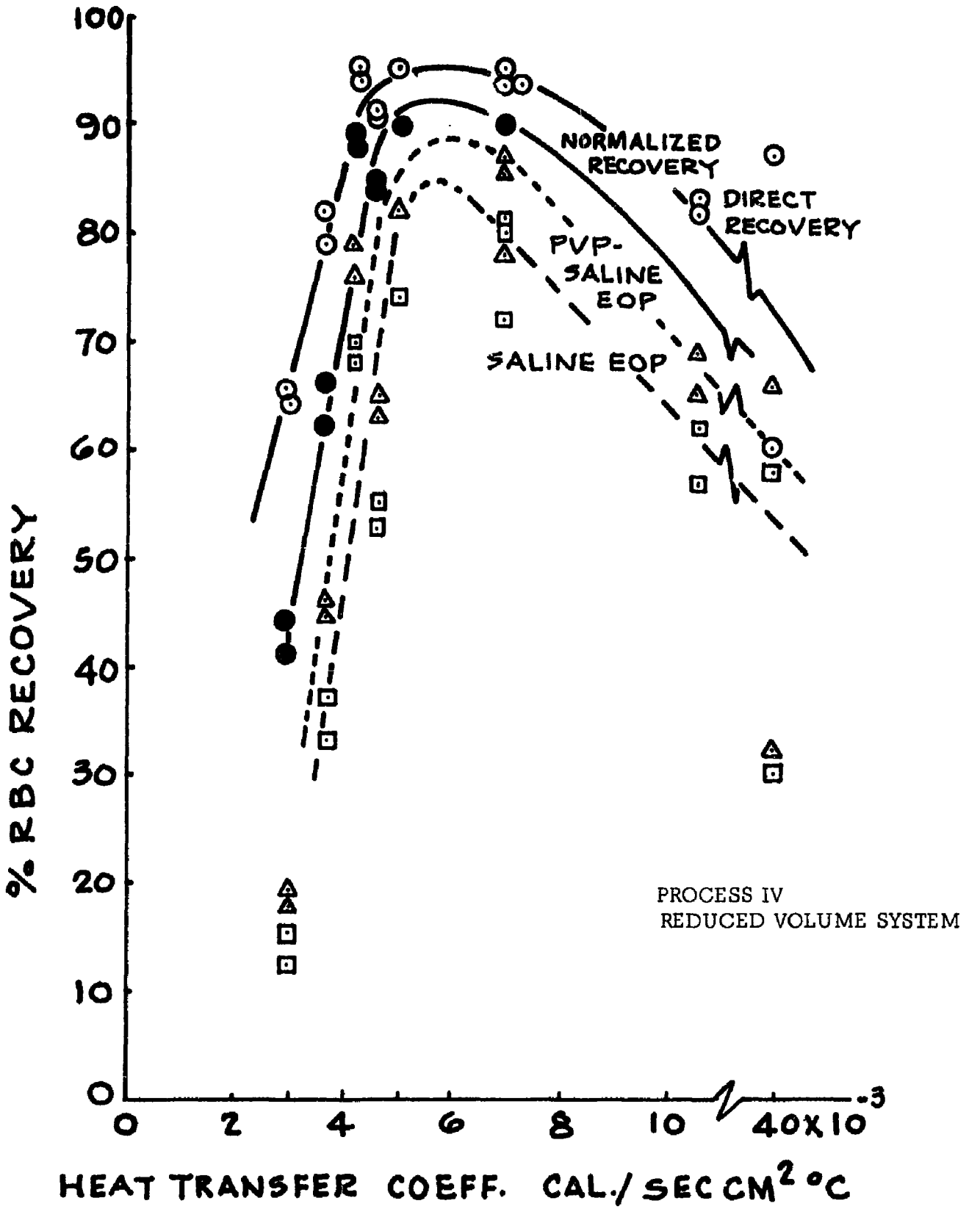

100
90
80
70
60
50
40
30
20
10
0
% RBC RECOVERY
NORMALIZED RECOVERY
DIRECT RECOVERY
PVP-SALINE EOP
SALINE EOP
PROCESS IV
REDUCED VOLUME SYSTEM
0
2
4
6
8
10
40X10-3
HEAT TRANSFER COEFF. CAL./SEC CM2 °C

TABLE IV-32

COMPARISON OF METAL AND PLASTIC CONTAINERS

FOR PROCESS - IV (PVP-SYSTEM)

Container	Direct % RBC Recovery	Resuspension Recovery % Saline	Resuspension Recovery % PVP
Metal	97(96)*	86	92
Plastic	93(93) *	87	93

* Values in parentheses are after normalizing hematocrit and reducing PVP conc. by dilution 5:8 with saline.

See footnotes Table IV-3.

Thawing was carried out at temperatures of 37°, 45°, and 55° with essentially equivalent results. Optimum conditions of agitation were observed at about 200 cycles/minute for cooling and 50-150 cycles/minute for thawing (Figure IV-20). Thawing has been carried out manually (75-100 cpm approximately) with recoveries as high as those obtained with mechanical agitation.

3. Process Development

Figure IV-21 shows the reduced volume PVP Process IV system in flow diagram. Only limited study of this system in large volumes has been done. Table IV-33 and IV-34 show results obtained on freezing pint units of blood by Process IV.

FIGURE IV-20

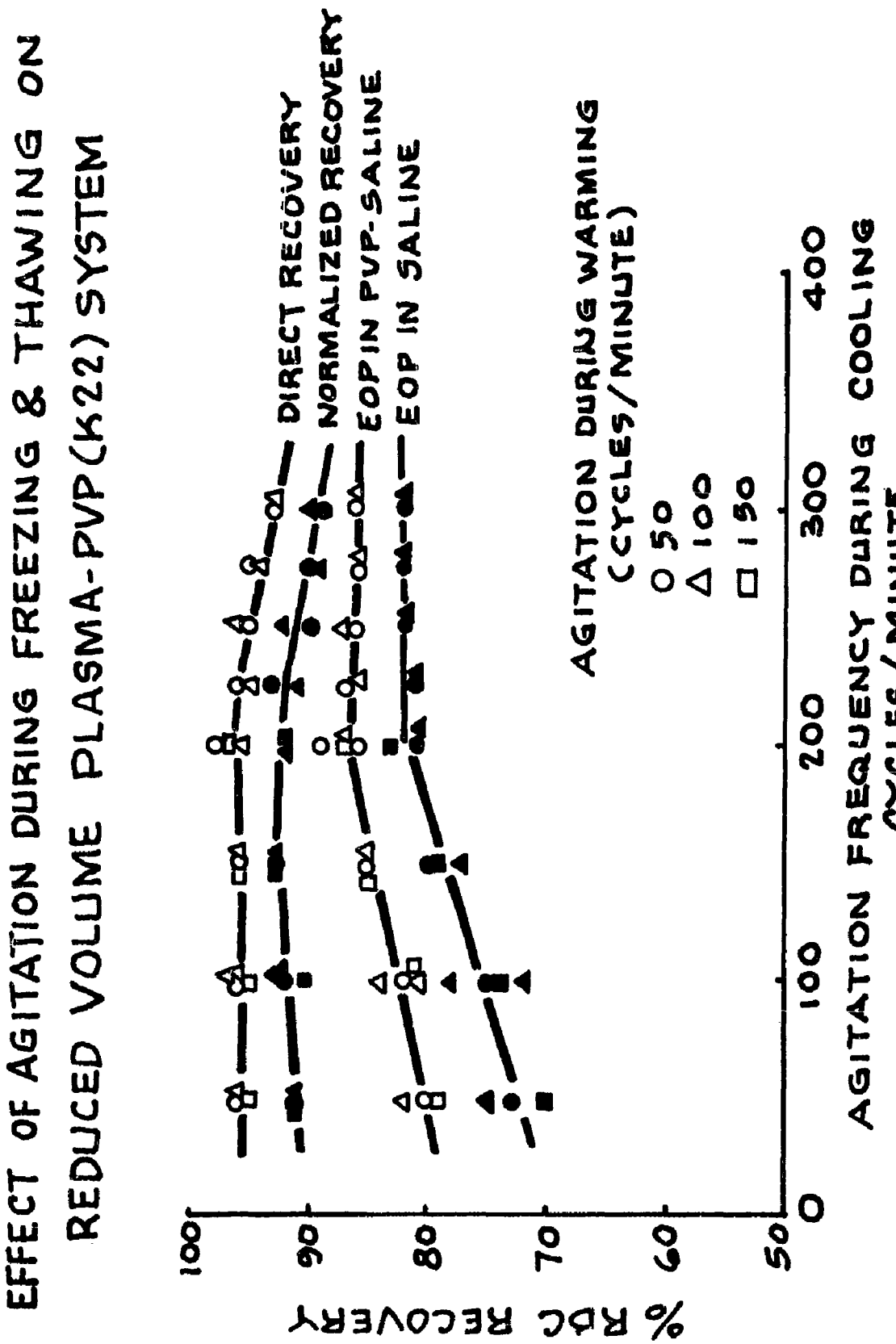

EFFECT OF AGITATION DURING FREEZING & THAWING ON REDUCED VOLUME PLASMA-PVP(K22) SYSTEM
% RBC RECOVERY
100
90
80
70
60
50
0
100
200
300
400
AGITATION FREQUENCY DURING COOLING CYCLES/MINUTE
AGITATION DURING WARMING (CYCLES/MINUTE)
○ 50
△ 100
□ 150
DIRECT RECOVERY
NORMALIZED RECOVERY
EOP IN PVP-SALINE
EOP IN SALINE

FIGURE IV - 21

FLOW SHEET FOR PROCESS - IV

REDUCED VOLUME SYSTEM USING PVP

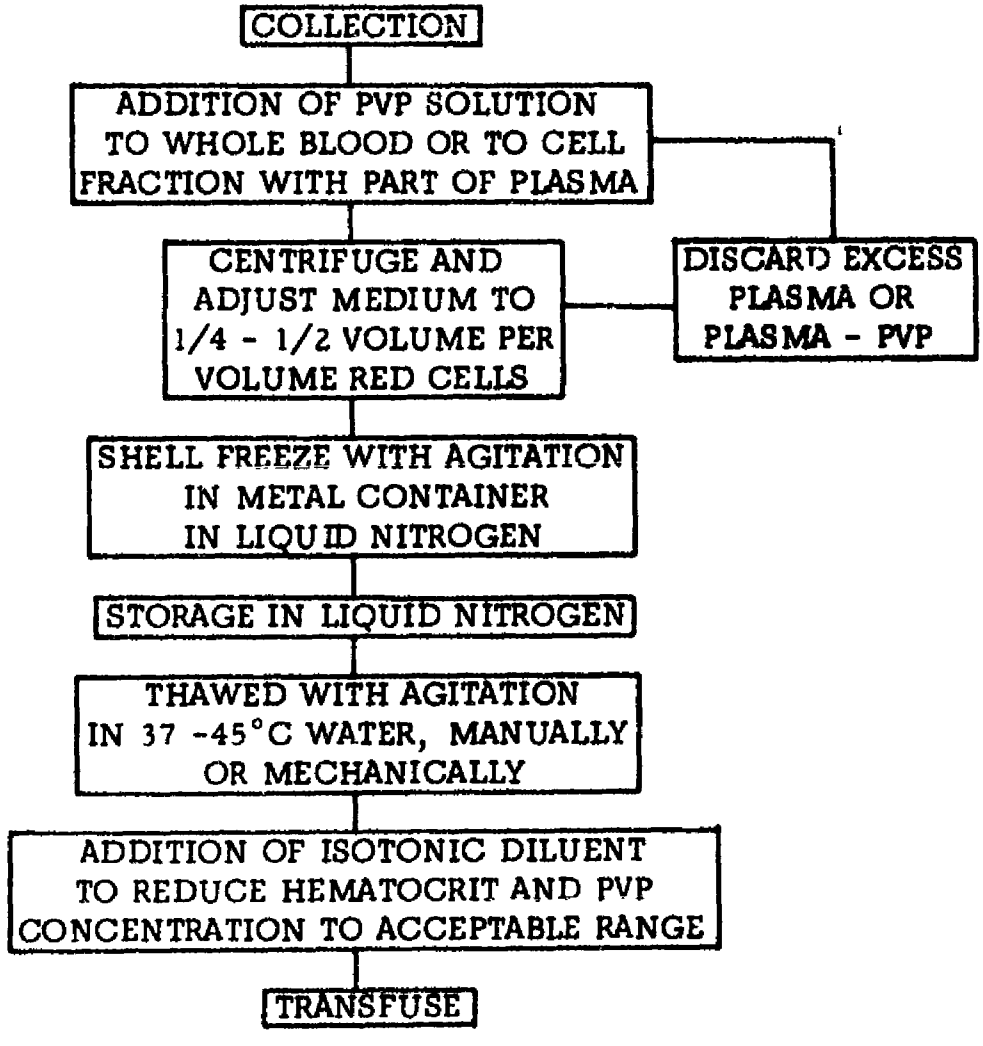


TABLE IV-33

PROCESSING OF PINT VOLUMES OF BLOOD

BY PROCESS - IV (REDUCED VOLUME PVP SYSTEM)

Exp. No.	% Direct RBC Recovery	% RBC Recovery After Dilution	% Resuspension Recovery Saline	% Resuspension Recovery PVP
1	92	88	71	78
2	95	90	84	91

Full pints (500-600 ml) of whole blood combined with 30% K30 PVP-0.05 M NaCl to give RBC suspended in medium of composition: 50% plasma- 15% PVP-0.1 M NaCl. The mixture was centrifuged and PVP-plasma removed by aspiration to leave 1/4 vol. medium per vol. RBC's. This mixture was frozen in PVP-MeOH coated XFC-1/2 metal containers (capacity: 450 ml approximately) with agitation at 200 cpm in liquid nitrogen. Thawing in water at 45°C at 150 cpm. Dilution 5:8 with isotonic saline after thaw. (Sample withdrawn for direct recovery before saline addition).

TABLE IV-34

PROCESSING OF PINT VOLUMES OF BLOOD

BY PROCESS - IV (PVP SYSTEM)

Exp. No.	% RBC Recovery	% RBC Recovery After Dilution	% Saline Resuspension Recovery
1	95	87	77

A pint (580 cc) of blood drawn into ACD-B was centrifuged and 240 cc of plasma removed. To the packed cells was added 40 cc of 30% Plasdone-C in 0.05 M NaCl and the mixture frozen in an uncoated FC-1 container with 200 cpm agitation. After thawing in a 45°C water bath at 160 cpm, it was diluted with 230 cc of physiological saline. (Sample withdrawn for direct recovery prior to saline addition).

TABLE IV - 35

Freeze-Thaw Studies with Fraction V (Cohn)
as the Protective Additive

Samples	% Fraction V	Volume Frozen (cc)	Container	% Direct RBC Recovery	PVP-Saline EOP
1	30	53	BFF 19110	93	82
2	30	53	"	93	80
3	20	53	"	93	81
4	20	53	"	91	79
5	7	38	BFT 3365	0	--
6	12.5	38	"	0	--
7	18	38	"	92	69
8	25	38	"	93	73

Resuspension: 1 volume of blood added to 9 volumes of isotonic 3.5% PVP (K-30) buffered to a pH of 7.4 and allowed to stand 2-4 hours.

Blood Preparation: Centrifuge citrated blood and remove plasma leaving 50% of the original volume as packed cells in residual plasma.

Samples 1-4: Add to cells equal volume of physiological saline containing given quantity of Fraction V.

Samples 5-8: Add to cells equal volume of physiological saline and Fraction V such that extra-cellular concentration of Fraction V is as given.

Freezing Condition: Blood in uncoated container agitated in liquid N_2.

Thawing Condition: Agitation in 45°C water bath.

B. Protection Based on Human Serum Albumin

Exploratory studies with small volumes of blood indicated that serum albumin afforded good protection to erythrocytes during freezing and thawing. Cohn's Fraction V was used for this purpose. Data are shown in Table IV-35. These results prompted further study, since it was assumed that a protein preparation of this type, well-established in medical use, would not require extensive pharmacological investigation as a transfusible substance. Further work showed, however, that, when albumin was diluted in plasma, red cell protection was less effective than when diluted in saline, Table IV-36. This was confirmed repeatedly.

TABLE IV-36

COMPARISON OF DILUENT FOR FRACTION V (Cohn)

Additive	% Direct RBC Recovery	PVP Saline EOP
In autologous plasma	88	62
In physiological saline	92	82

Resuspension: See Table IV-35

Blood Preparation: Citrated blood centrifuged and plasma removed leaving 50% of initial volume as concentrated red cells. An equal volume of additive was readded such that the extracellular concentration of added Fraction V was 25%.

Volume Frozen: 38 cc

Container: Uncoated BFT 3365

As a practical matter, serum albumin, because of its expense, would have to be used sparingly. It was decided that not more than twice the quantity of albumin normally present in a unit of collected blood would be used to provide freeze-thaw protection. To achieve the necessary protective concentration required that cells be processed in a small volume of suspending medium. On thawing, the preparation could be diluted to a normal hematocrit and a normal protein level with saline solution. As with Process IV, described earlier, post-thaw handling would involve a single, simple operation.

Further study showed that when the hematocrit before freezing exceeded 60% in the albumin-containing systems, red cell recovery was adversely affected (Figure IV-22). It was also found that a minimum concentration of 16% albumin in the suspending medium was necessary for maximum protection, the actual amount of albumin equalling that present in the volume of blood from which the cells were obtained. See Table IV-37 and Figure IV-23. Also, as indicated in Figure IV-24 (and IV-22) the total amount of albumin present (another way of regarding the volume of medium in which the cells are suspended) influences recovery.

These findings led to more intensive investigation of the utility of albumin as an additive in a low temperature blood preservation process.

1. Chemical Parameters

For in vitro studies and optimization both Fraction V, a plasma-protein fraction consisting primarily of albumin, and clinical serum albumin were used. The American National Red Cross supplied Fraction V in powder form and clinical serum albumin in a 25% salt-poor solution. This maximum clinical albumin concentration of 25% placed a practical limit on the additive concentration. Most important it was found that electrolyte concentration in the albumin solution varied with each lot. Hence, a sample of each was tested to determine the additional amount of NaCl required to give maximum freeze-thaw cell recoveries. One such optimization curve is shown in Figure IV-25.

For each series of clinical studies salt and pyrogen-free water were added to a fresh bottle of clinical serum albumin. The resulting mixture was sterilized.

In specifying the quantity of albumin used in a blood preparation, we generally use the term "albumin factor (A. F.)." This is the ratio of the quantity of albumin used to protect a given number of red cells to the quantity of albumin normally associated with these cells in vivo. An average concentration of 2.4 gm of albumin per 100 cc of blood collected with ACD-B was used as the norm.

FIGURE IV-22

EFFECT OF PRE-FREEZE HEMATOCRIT ON RED CELL RECOVERY:
25 GRAMS OF FRACTION V ADDED PER 100 CC. OF PLASMA

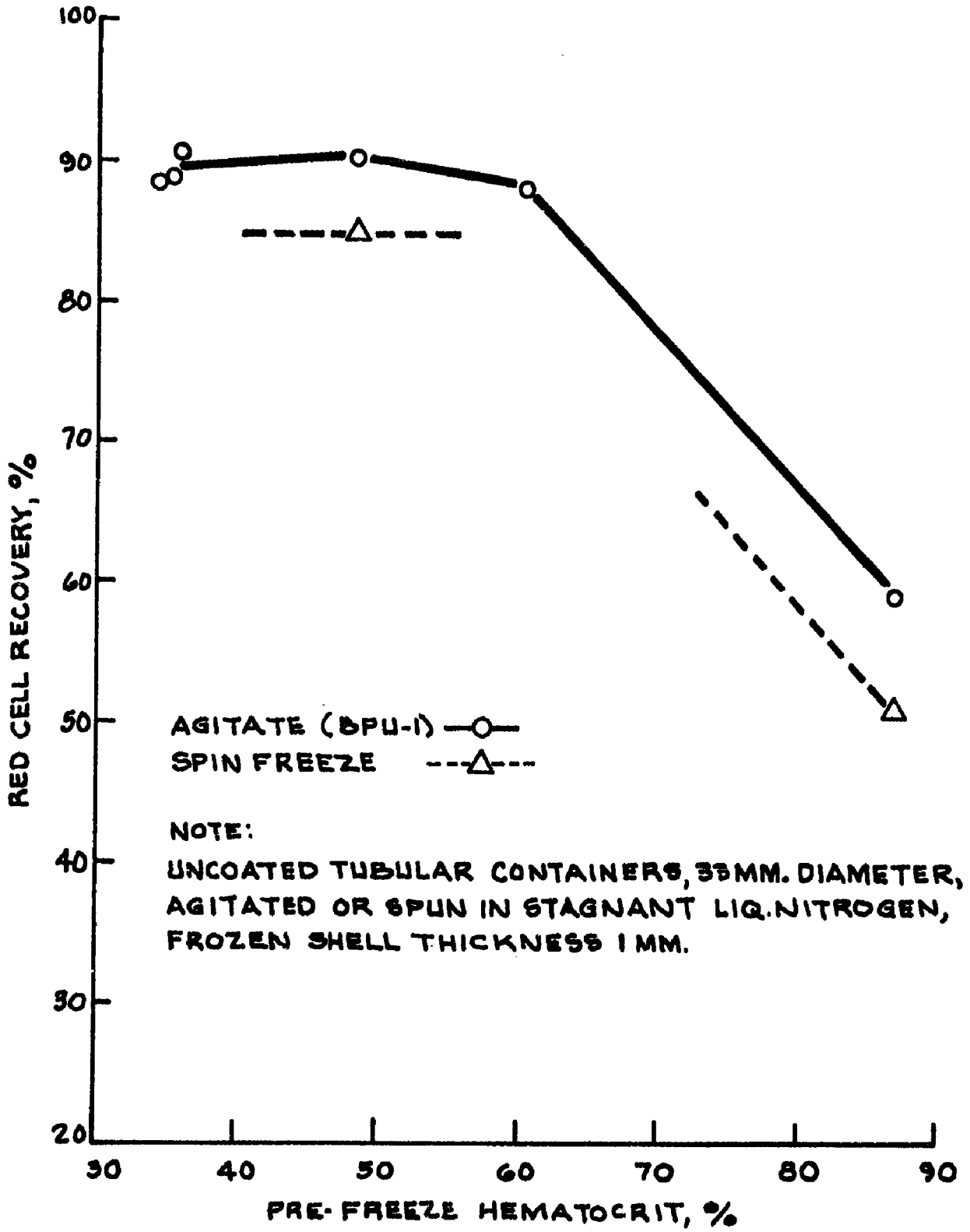


TABLE IV-37

RECOVERY OF ERYTHROCYTES FROZEN AND THAWED WITH A QUANTITY OF ALBUMIN EQUIVALENT TO THAT REMOVED WITH PLASMA

Albumin Concentration in Additive (%)	Volume Additive per 4.5 cc Cells (cc)	Prefreeze Hematocrit (%)	PVP-Saline EOP (%)
40	0.69	66.0	89
35	0.79	68.0	88
30	0.92	64.0	90
25	1.10	62.0	88
20	1.38	59.0	86
16	1.72	53.5	82
10	2.75	47.3	55
Whole Blood	---	37.5	27
Packed Cells	---	85.5	40

Cells: Blood in ACD-B from which 55% of the volume was removed as plasma.

Additive: Fraction V in physiological saline.

Volume Frozen: 5 cc in 5 mm aluminum envelopes.

Recovery: Analysis made after 15 minutes standing of the mixture of 1 volume of blood and 9 volumes of isotonic 3.5% PVP medium.

FIGURE IV-23

RECOVERY OF ERYTHROCYTES FROZEN AND THAWED WITH A QUANTITY OF ALBUMIN EQUIVALENT TO THAT NORMALLY SURROUNDING AN ERYTHROCYTE

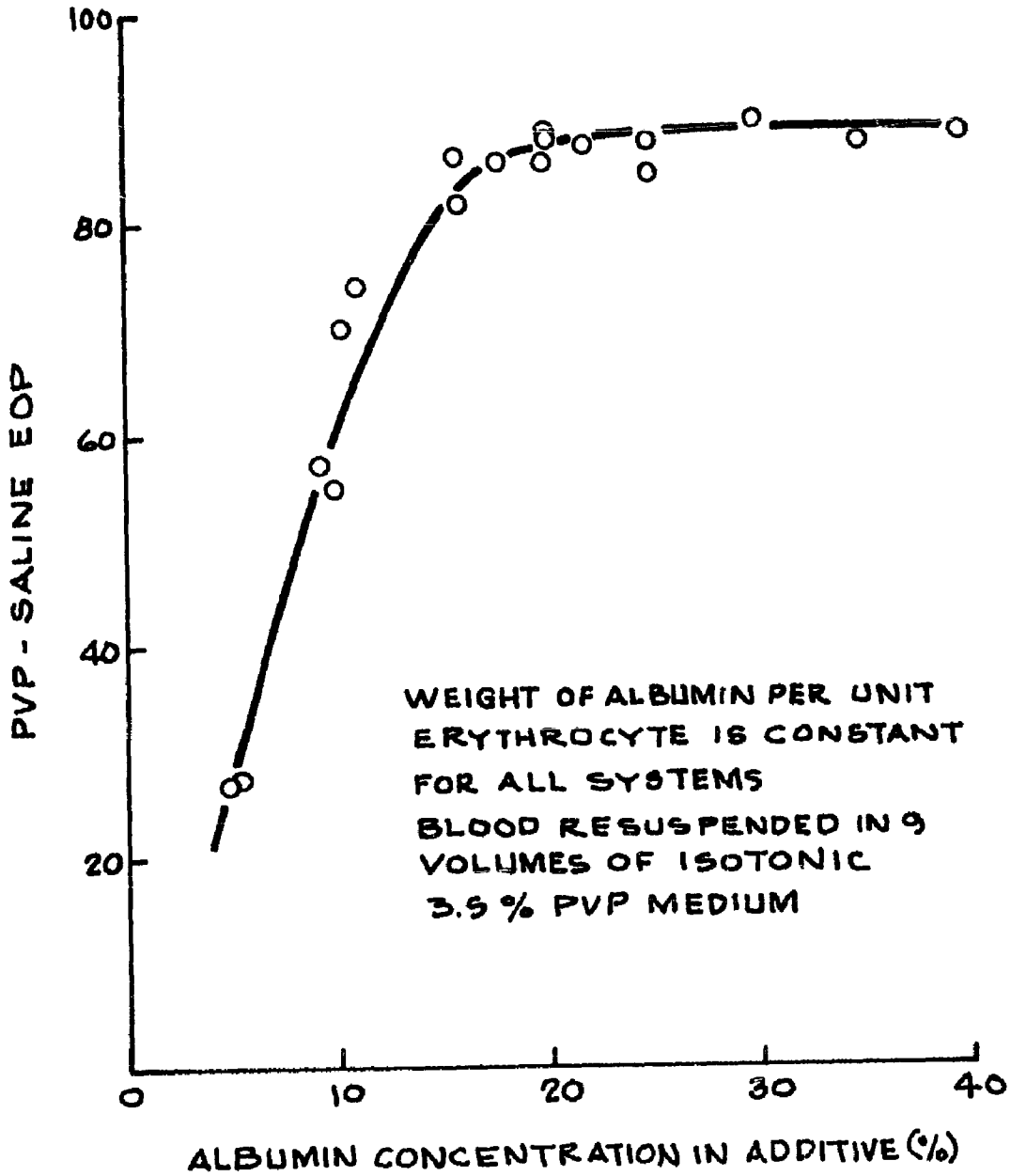


FIGURE IV-24

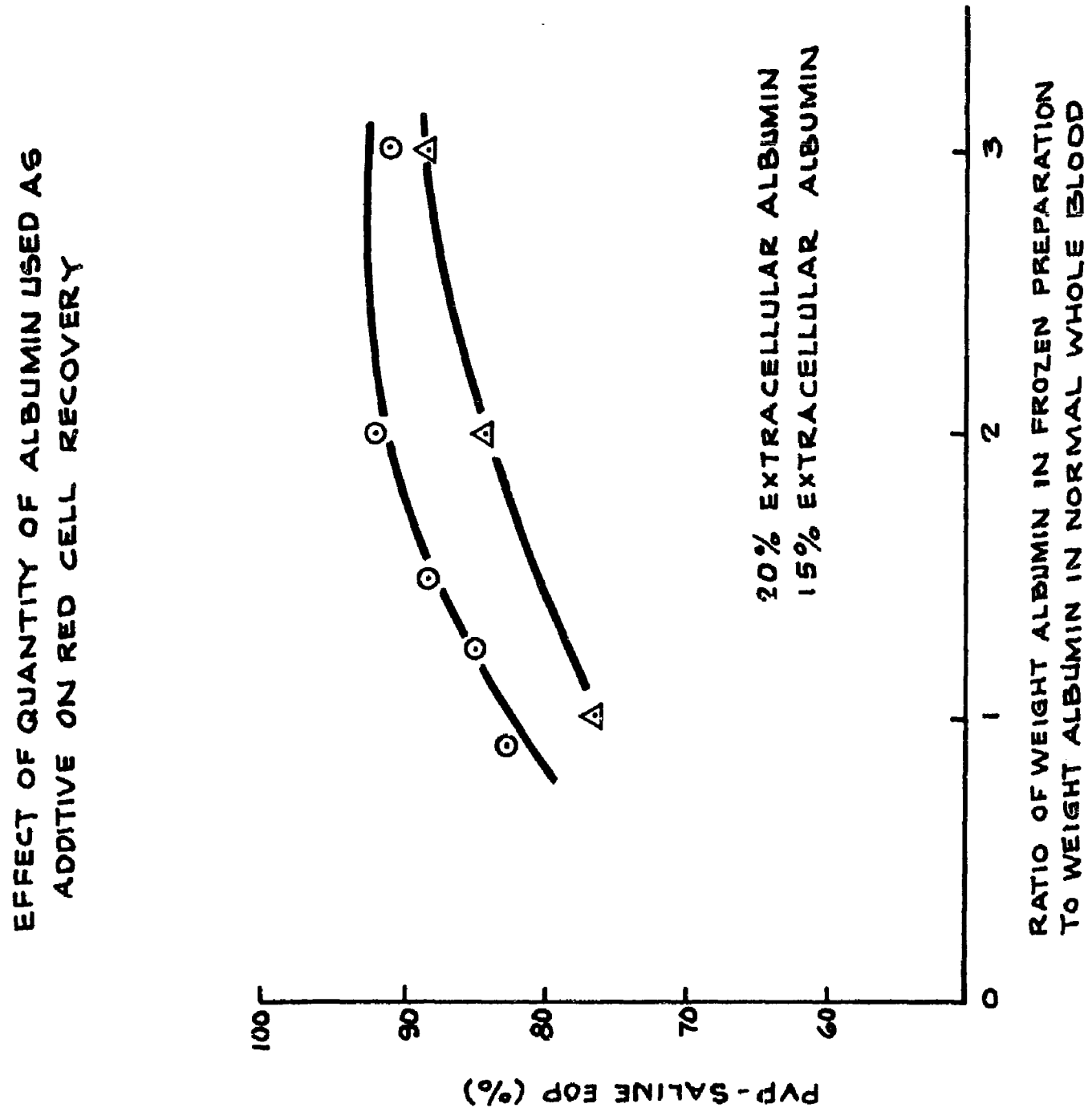
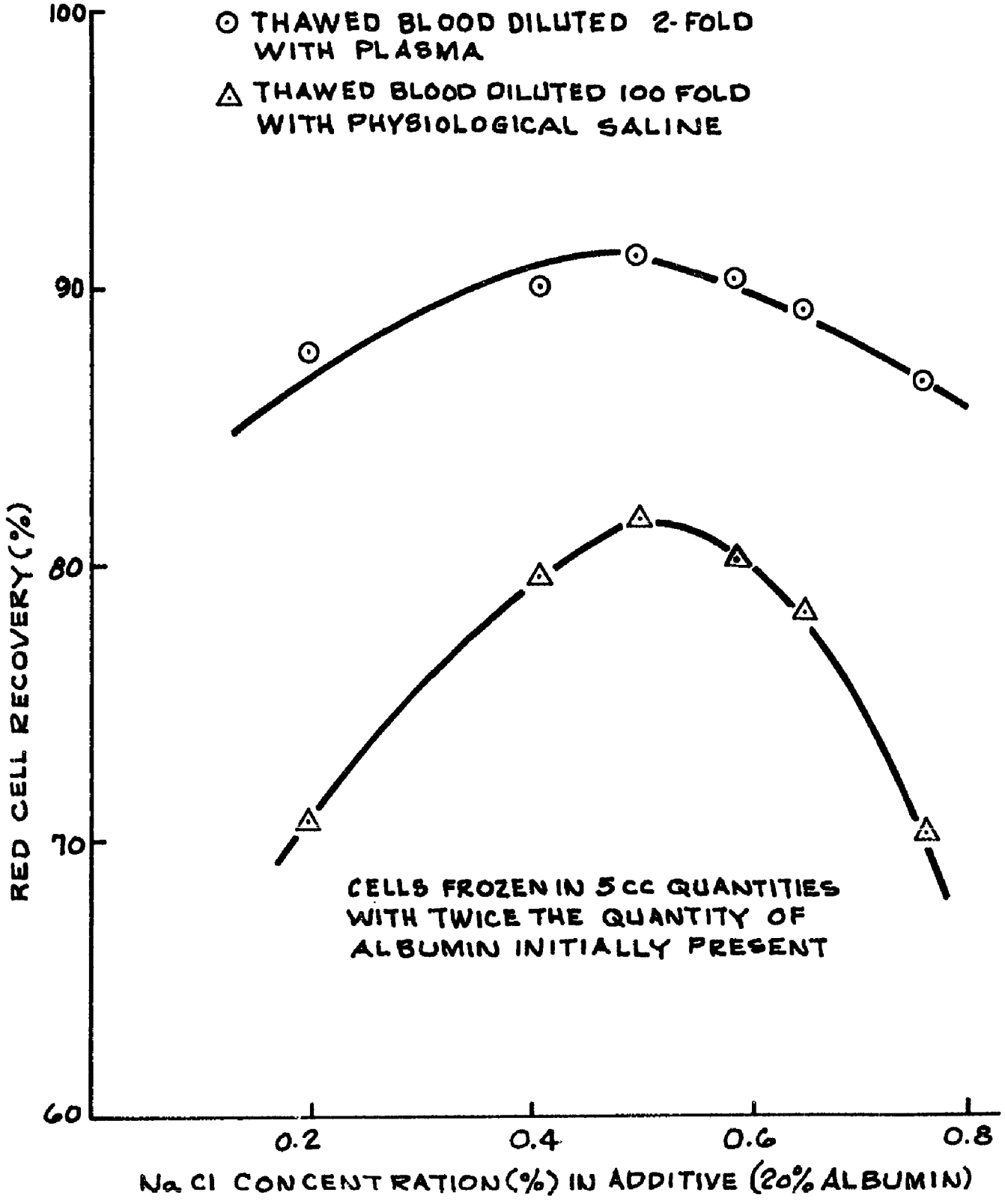

EFFECT OF QUANTITY OF ALBUMIN USED AS
ADDITIVE ON RED CELL RECOVERY
20% EXTRACELLULAR ALBUMIN
15% EXTRACELLULAR ALBUMIN
PVP - SALINE EOP (%)
100
90
80
70
60
0
1
2
3
RATIO OF WEIGHT ALBUMIN IN FROZEN PREPARATION
TO WEIGHT ALBUMIN IN NORMAL WHOLE BLOOD

FIGURE IV-25

ELECTROLYTE OPTIMIZATION OF CLINICAL SERUM ALBUMIN
(Lot*1742)



A study of erythrocytes frozen and thawed in the presence of Fraction V showed that the cell volumes increased during the process and continued to increase during post-thaw storage. On the assumption that some reversible membrane damage had occurred, similar to that found in stored blood (4°C), it was decided to attempt a reversal of this cellular damage by the addition of nucleosides. The regenerative activity of nucleosides on stored blood is reported in the literature (11).

Survey experiments with inosine showed that this compound did indeed improve the osmotic stability of cells frozen and thawed with albumin, if the thawed cells were incubated at 37°C after thawing for a period of 1/2- 1 hour. See Table IV-38. It can be seen that part of the freeze-thaw damage to cells is reversible. The effect of additive pH on cell recovery was tested both with and without incorporation of inosine. Although the effect of pH was inconclusive there were indications that inorganic phosphate was beneficial and that a combination of inosine and inorganic phosphate was definitely detrimental. Post-thaw incubation of cells protected with phosphate-buffered additive showed increases in resuspension stability. Direct comparison studies were made and as Table IV-39 shows, the use of phosphate alone gives superior cell resuspension stabilities.

Experiments were also conducted with cholesterol and lecithin. The concentrations of lecithin and cholesterol in the 20% Fraction V-saline solutions were 0.042% and 0.084% respectively. Results of tests conducted essentially as described for inosine in Table IV-38 showed no beneficial effects of these compounds.

Inorganic phosphates not only increased the resuspension stabilities of red cells, when subjected to a period of incubation following thawing, but also improved the storage stability (4°C) of the cells after thawing. Fraction V was dissolved in various mixtures of physiological saline and isotonic phosphate solution, as shown in Table IV-40, to optimize the ratio of buffer solution to saline (to enhance the storage stability of thawed blood). As shown, only a small quantity of the phosphate is necessary and without phosphate extensive post-thaw hemolysis occurred.

In the experiments reported thus far, 1:10 PVP-saline EOP's consistently above 90% and 1:100 saline EOP's above 80% were possible only by washing the erythrocytes prior to freezing with a dilute solution of Fraction V or with the additive. To reduce this prefreeze handling of the blood a volume of diluent was added to the ACD-B blood which was then centrifuged at a force of 12,000 x G to assist in the packing of cells and thus in the removal of plasma.

Diluents used were solutions of physiological saline, 10% lactose, 0.2 M glucose in physiological saline and an isotonic phosphate buffer. To the diluents were added equal volumes of blood and, after mixing and equilibration,

TABLE IV-38

INOSINE IN 20% FRACTION V: COMPARISON WITH ADDITIVE WITHOUT INOSINE

Post-Thaw Equilibration at 37°C (hrs.)	PVP-Saline EOP (%) With Inosine	No Inosine
0	81.7	81.6
1/2	82.4	75.6
1	83.3	75.5
1 1/2	82.6	74.5

Additive: 20% Fraction V in physiological saline with 0 or 1.68% inosine.

Blood Mixture: 5 cc packed cells (washed with 5% Fraction V) with 1.56 cc additive.
Volume Frozen: 5 cc in 5 mm aluminum envelopes.

Normalization: To 1 cc of the thawed sample equilibrated for the period given was added 0.5 cc of normal saline.

Resuspension: 1 volume normalized blood added to 9 volumes of isotonic 3.5% PVP medium and allowed to stand 15 minutes prior to analysis for free hemoglobin.

TABLE IV-39

COMPARATIVE EFFECTS OF INOSINE AND INORGANIC PHOSPHATE ON FREEZE-THAW RECOVERY OF ERYTHROCYTES

Additive	37°C Incubation Before Normalization (hrs.)	Resuspension EOP of Normalized Blood (%) A	B
1	0	87.7	77.0
	1/2	89.0	80.8
	1	88.9	80.6
2	0	76.6	62.0
	1/2	84.2	67.3
	1	82.7	65.5
1	1/2	91.1	84.3
2	1/2	87.5	79.8

Additive:

1. 15% Fraction V in a solution of 3 parts physiological saline and 2 parts isotonic phosphate buffer.

2. 15% Fraction V, 1.68% Inosine in physiological saline.

Phosphate Buffer: 11.32 gm $NaH_2PO_4 \cdot H_2O$ and 16.25 gm $Na_2HPO_4 \cdot 12\ H_2O$ in 1 liter of solution.

Normalization: The addition of 0.34 cc physiological saline to 1 cc of blood preparation in order toreduce extracellular protein concentration to normal levels.

Resuspension: Analysis for free hemoglobin conducted 1/2 hour after addition of blood to resuspension media.

A: 10-fold dilution in isotonic 3.5% PVP solution.

B: 100-fold dilution in physiological saline.

TABLE IV-40

THE EFFECT OF VARIOUS CONCENTRATIONS OF PHOSPHATE BUFFER[1] IN A 20% FRACTION V SOLUTION[2] ON THE POST-THAW STABILITY OF RED CELLS

Conc. by Vol. of Phosphate Solution Present in Fraction V Solution (%)	PVP-Saline EOP after Thawing and 30 Min. Incubation at 37°C (%)	PVP-Saline EOP of Thawed Blood after Storage for 20 hrs. at 4°C (%)
20.0	91.6	90.1
	91.9	90.4
15.0	92.0	91.2
10.0	92.3	90.6
5.0	91.5	89.9
	92.3	90.5
0.	93.1	81.9
20.0[3]	94.5	91.8

Blood Preparation: Packed cells obtained by centrifugation of 10 cc of ACD-B blood at 12,000 x G for 10 minutes were treated with a volume of appropriate additive solution. After mixing and centrifuging, a volume of supernatant was removed leaving the cells in a solution of albumin equal to 1.5 times the quantity normally surrounding the cells.

Processing Conditions: Small volumes (0.8 cc) in containers of 5 mm cross-section frozen by immersion in liquid nitrogen and thawed by immersion in water at 37°C.

Resuspension: One volume of thawed blood suspended in 9 volumes of buffered PVP (3.5%) for period of 30 minutes.

(1) Phosphate Buffer: 11.32 gm $NaH_2PO_4 \cdot H_2O$ and 16.25 gm $Na_2HPO_4 \cdot 12H_2O$ in 1 liter of solution.

(2) Four volumes of 25% Fraction V in physiological saline diluted with 1 volume of an isotonic phosphate - saline mixture.

(3) Mix prepared by washing the packed cells from a 10 cc volume of ACD-B blood 3 times with the appropriate additive solution.

the mixtures were centrifuged and the supernatant poured off. A predetermined volume of 25% Fraction V solution was added to each of the packed cell samples and 5 cc of the resultant mixture frozen and thawed stagnantly in 5-mm aluminum envelopes. Resuspension EOP's for all samples were approximately 90% for a 10-fold dilution in 3.5% PVP solution and 70-75% for a 100-fold dilution in physiological saline.

The results suggested the possible feasibility of this process. In subsequent studies an essentially extracellular material, mannitol, was used as the blood diluent.

Red cell volumes may be altered by the addition of mannitol solutions to whole blood. By judiciously selecting appropriate concentrations of mannitol it is possible to control the hematocrit of the blood-mannitol mixture and, hence, the amount of supernatant removable on centrifugation. In this manner it is possible to prepare erythrocyte-albumin mixtures of varying extracellular protein concentration by adding to erythrocytes treated with mannitol a stock solution of concentrated human serum albumin.

For the preliminary studies with mannitol one volume of ACD-B blood was added to an equal volume of the diluent containing from 1.0% to 12.5% mannitol in distilled water. The resultant mixtures were centrifuged at a force of 12,000 x G and the supernatant removed. The diluents containing less than 2.5% mannitol caused cells to hemolyze. To each of the remaining packed-cell units was added a volume of 25% solution of albumin equivalent to a quantity of albumin approximately 1.5 times that removed with the plasma. According to approximate calculations the extracellular concentration of albumin in the mixtures to be frozen ranged from 13 to 22%.

Five-cc samples were frozen and thawed stagnantly in 5-mm aluminum envelopes and incubated for 30 minutes at 37°C. Aliquots of the blood were then diluted 10 fold in isotonic 3.5% PVP and 100 fold in physiological saline. The differences in cell recovery were slight; however, the best results occurred with the use of 5% mannitol for which EOP's in PVP and saline were 92% and 79% respectively.

With 5% mannitol solution and high speed centrifugation (12,000 x G), as a means for separating plasma from cells, several tests were run to determine the optimum concentration of phosphates required in the albumin additive to provide maximum freeze-thaw protection and post-thaw stability for the cells. Solutions of physiological saline, isotonic phosphate buffer, and combinations of both, containing 25% Fraction V were used as additives. The results presented in Table IV-41 indicate that only a small amount of phosphate is necessary to provide maximum recovery and stability of frozen and thawed cells. Although not tabulated these experiments reconfirmed the point that a post-thaw incubation period of 30 minutes at 37°C improved cell stability on resuspension.

TABLE IV-41

BLOOD TREATMENT WITH 5% MANNITOL SOLUTION: DETERMINATION OF PHOSPHATE REQUIREMENTS IN THE FRACTION V ADDITIVE

Volume Ratio in Additive Saline: Phosphate Buffer (Isotonic)	In Vitro Age of Blood (days)	Efficiency of Process (%)			
		Saline		3.5% PVP	
		Immediate	Overnight (Storage at 4°C)	Immediate	Overnight (Storage at 4°C)
5:0	2	81.5	75.5	94.4	92.8
4:1	2	80.0	77.1	93.7	92.3
3:2	2	79.2	75.0	93.4	92.0
2:3	2	76.8	73.4	92.4	89.9
1:4	2	76.8	72.4	92.3	90.3
0:5	2	75.8	73.0	91.5	89.2
5:0	3	83.5	79.7	93.7	92.7
9:1	3	81.0	79.2	93.9	94.2

Blood Dilution: 1 volume ACD-B blood with 1 volume 5% mannitol

Additive: 25% Fraction V in isotonic solutions with physiological saline:isotonic phosphate buffer ratios as tabulated. A volume of additive containing approximately 1.4 times the albumin removed from the cells with the plasma was added to the packed cells.

Phosphate Buffer: 11.32 gm $NaH_2PO_4 \cdot H_2O$ and 16.25 gm $Na_2HPO_4 \cdot 12\ H_2O$ in 1 liter of solution.

Processing Conditions: 5 cc volumes in 5 mm aluminum envelopes frozen by immersion with gentle agitation in liquid nitrogen and thawed by immersion with gentle agitation in water at 45°C for 90 sec. followed by incubation at 37°C for 1/2 hr.

Saline: 100-fold dilution in physiological saline.

3.5% PVP: 10-fold dilution in isotonic 3.5% PVP solution.

All analyses 1/2 hour after dilution of thawed blood.

Further experiments were conducted to determine whether or not other diluent fluids could be used as successfully as mannitol. Samples of blood drawn into ACD-B anticoagulant were diluted with equal volumes of 5% mannitol, physiological saline or isotonic phosphate buffer of pH 6.4 prior to centrifugation of the samples at 12,000 x G for plasma separation. A volume of 25% Fraction V solution containing 1.4 times the quantity of albumin removed with the plasma was used as the protective additive. Portions of the blood samples (frozen in 5-cc quantities) were resuspended in 9 volumes of isotonic 3.5% PVP solution and in 99 volumes of physiological saline immediately after thawing and after 1/2-hour incubation of the thawed blood at 37°C. The thawed samples, both with and without incubation, were then stored at 4°C overnight and aliquots of each again resuspended. In all cases the incubated samples gave better recoveries. These results are tabulated in Table IV-42. The evidence indicates that a major portion of the plasma of whole blood should be removed prior to addition of albumin to attain good recoveries of frozen and thawed cells. It is possible that saline or buffer concentrations could be optimized to provide cell recoveries equivalent to that provided with mannitol. However, the use of 5% mannitol and additive prepared in physiological saline gave the best immediate results. Hence, this system was used as a control in many of the subsequent tests to improve yields and study methods for freezing and thawing 30-50 cc samples of blood. In Table IV-43 are presented the resuspension recoveries of the cells frozen and thawed in this control system during a period of one month. For blood drawn into ACD-B or ACD-A the results were quite reproducible. Blood drawn through an ion-exchange column to prevent coagulation is not stable <u>in vitro</u> and this instability is reflected in the freeze-thaw and resuspension yields of intact red cells.

The potential use of mannitol solutions to dehydrate erythrocytes, which would permit the use of salt-poor additives, was investigated. As a preliminary experiment whole blood was added to equal volumnes of mannitol solutions ranging in concentration from 10 to 15%. Each of these mixtures was divided into 5 equal aliquots which were then centrifuged and the supernatant removed. To each aliquot of packed cells was added a quantity of distilled water or saline containing up to 0.85% NaCl, equivalent to that volume of 25% Fraction V additive which would be used to preserve the cells. Subsequent observations showed that the colors of the supernatants of the various samples were equivalent, thus indicating the feasibility of using hypotonic additives without hemolyzing cells prior to freezing.

Additive solutions containing 25% Fraction V and 0-5% mannitol were then prepared in distilled water. Blood drawn into ACD-B was added to an equal volume of 15% mannitol, allowed to equilibrate, then centrifuged. The packed cells were resuspended in the various additives, then frozen and thawed in 5-cc sample quantities. The over-all hemolysis, after a 100-fold dilution of

TABLE IV-42

COMPARISON OF DILUENT FLUIDS

Whole Blood Diluent	Additive	Efficiency of Process (%) 1:9 PVP Immediate	1:9 PVP Overnight (Storage at 4°C)	1:99 Saline Immediate	1:99 Saline Overnight (Storage at 4°C)
A	1	94	93	83.5	80
A	2	94	94	81	79
B	1	92	91.5	80	78
B	2	93	91.5	81	78
C	1	93	91	80.5	78
C	2	92	91	80	78

Diluent: 1 volume blood, drawn into ACD-B, was added to 1 volume diluent prior to centrifugation at 12, 000 x G.

A - 5% mannitol
B - physiological saline
C - isotonic phosphate buffer of pH = 6.4

Additive: 2 cc for cells from initial 15 cc of ACD-B blood

1 - 25% Fraction V in physiological saline
2 - 25% Fraction V in a solution of 9 volumes saline and 1 volume phosphate buffer

Processing Conditions: 5 cc volumes in 5 mm aluminum envelopes frozen by immersion with gentle agitation in liquid nitrogen and thawed by immersion with gentle agitation in water at 45°C for 90 sec. followed by incubation at 37°C for 1/2 hour.

Resuspension: Dilutions (blood:medium) as shown in isotonic 3.5% PVP and physiological saline. Analysis for free hemoglobin 1/2 hour after resuspension.

TABLE IV - 43

Recoveries of Red Cells Packed Using Mannitol and Frozen and Thawed with 25% Fraction V in Saline

Anticoagulant	In Vitro Age of Blood(days)	Efficiency of Process (%) in PVP	in Saline
ACD-B	2	94.4	81.5
* Ion Exchange	1	88.6	79.1
* Ion Exchange	2	84.7	61.0
ACD-B	3	93.7	83.5
"	5	94.6	84.0
"	1	94.5	84.2
"	1	95.7	86.2
"	1	94.7	84.0
ACD-A	0	94.0	81.4
ACD-B	1	94.8	82.5
ACD-A	3	95.0	83.5

* Ion Exchange blood had as additive an amount of albumin equal to 1.1 instead of 1.4 times that quantity removed in the plasma. A volume of blood was added to an equal volume of 5% mannitol before centrifugation.

Samples frozen stagnantly in liquid N_2 in 5 cc quantities, and thawed by immersion with gentle agitation in water at 45°C, followed by incubation for 1/2 hour at 37°C.

Resuspension Recovery: Thawed blood diluted with resuspension media and analyzed for free hemoglobin after 1/2-hour standing.

PVP: 10-fold dilution with isotonic 3.5% PVP.

Saline: 100-fold dilution with physiological saline.

the thawed samples with physiological saline, was 40±3%; after a 10-fold dilution with isotonic 3.5% PVP solution, 13±2%, except where Fraction V without mannitol was used as the protective additive. In that experiment corresponding hemolysis values of 61 and 27%, respectively, were noted.

Although this particular experiment did not produce satisfactory levels of cell recovery, it demonstrated the fact that extracellular concentrations of albumin (or other extracellular polymeric additives) can be increased above the concentration level of the stock additive solution. By pretreating red cells with hypertonic mannitol solution, it is possible to reduce the cell volume nearly 50%. On resuspension in the albumin additive solution these cells regain much of their original volume, thus concentrating the extracellular protective material. In this manner it is possible to achieve calculated extracellular concentrations of albumin as high as 62% by employing a 25% stock additive solution of this material.

Subsequent tests were concerned with utilizing mannitol directly in the anticoagulant and decreasing the volume of mannitol used, to make bulk processing more feasible. The use of mannitol or other diluents in the anticoagulant gave results inferior to those using 5% mannitol after collection. In all processing techniques concentrations of mannitol other than 5% gave inferior in vitro recoveries when blood collected into ACD-B was frozen and thawed. Figure IV-26 shows in flow sheet form the reduced-volume-albumin Process IV systems used.

2. Process Development

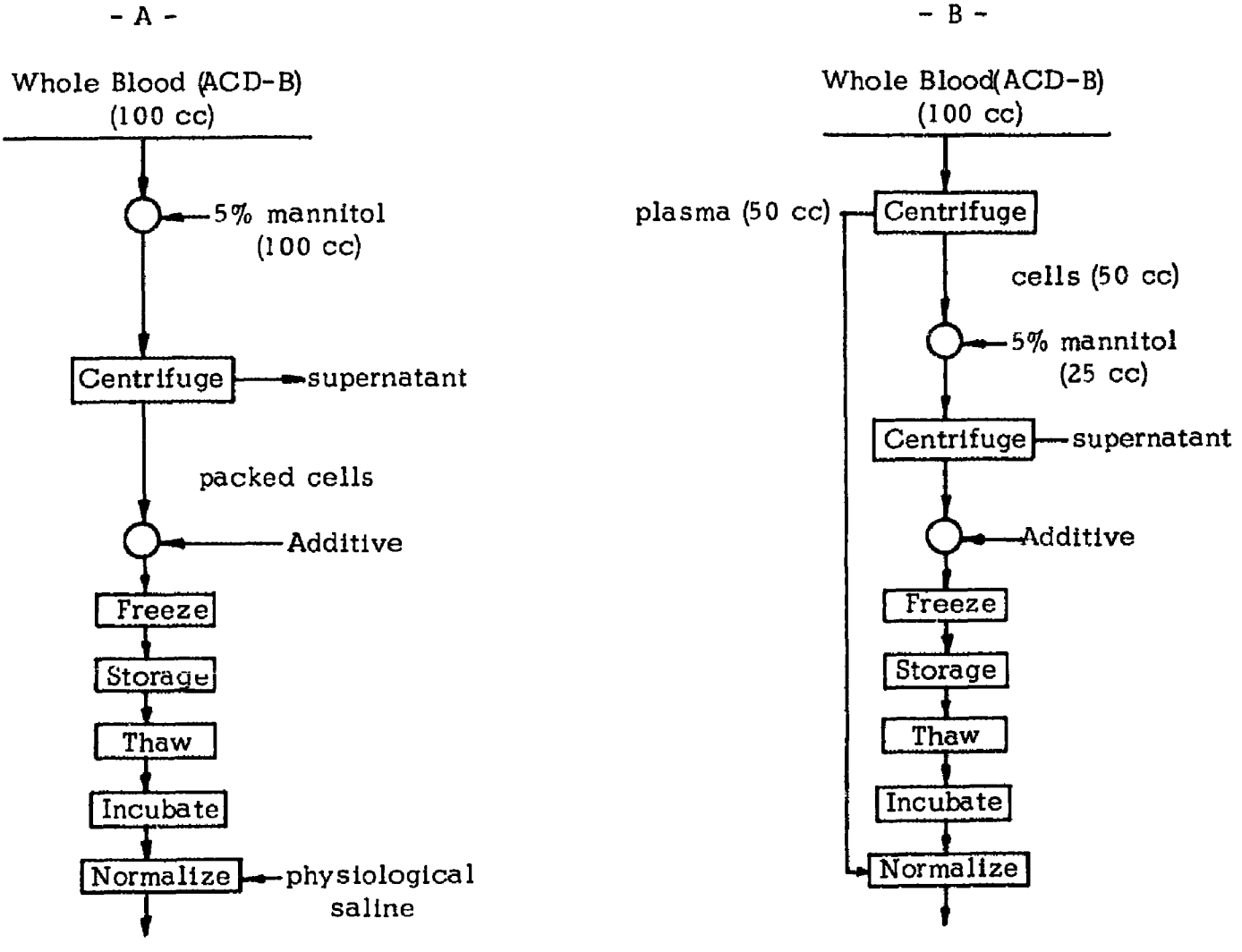
To freeze and thaw quantities of blood sufficient for clinical evaluation, with in vitro recoveries equivalent to those attained in 5 cc volumes, was the next objective. Two methods of freezing were evaluated. In the first, 30 cc of the high hematocrit blood preparations were frozen in rectangular aluminum containers of 75 cc capacity with agitation in the BPU-1; in the second 22 cc were frozen in a 33 mm cylindrical container rotating about its axis at about 1800 rpm. The container and equipment for "spin freezing" are shown in Figures IV-27 and IV-28.

After extensive heat transfer studies it was found that a wide range of cooling conditions were possible by using a liquid bath at a preset temperature and by spraying the container with liquid nitrogen, or a mixture of liquid and gaseous nitrogen. For BPU-freezing, cooling rates were controlled with container coatings.

A resume of the relationship between initial heat flux and red cell recovery of albumin preserved cells for the two methods of freezing is presented

FIGURE IV - 26

PROCEDURES FOR PROCESS IV USING ALBUMIN

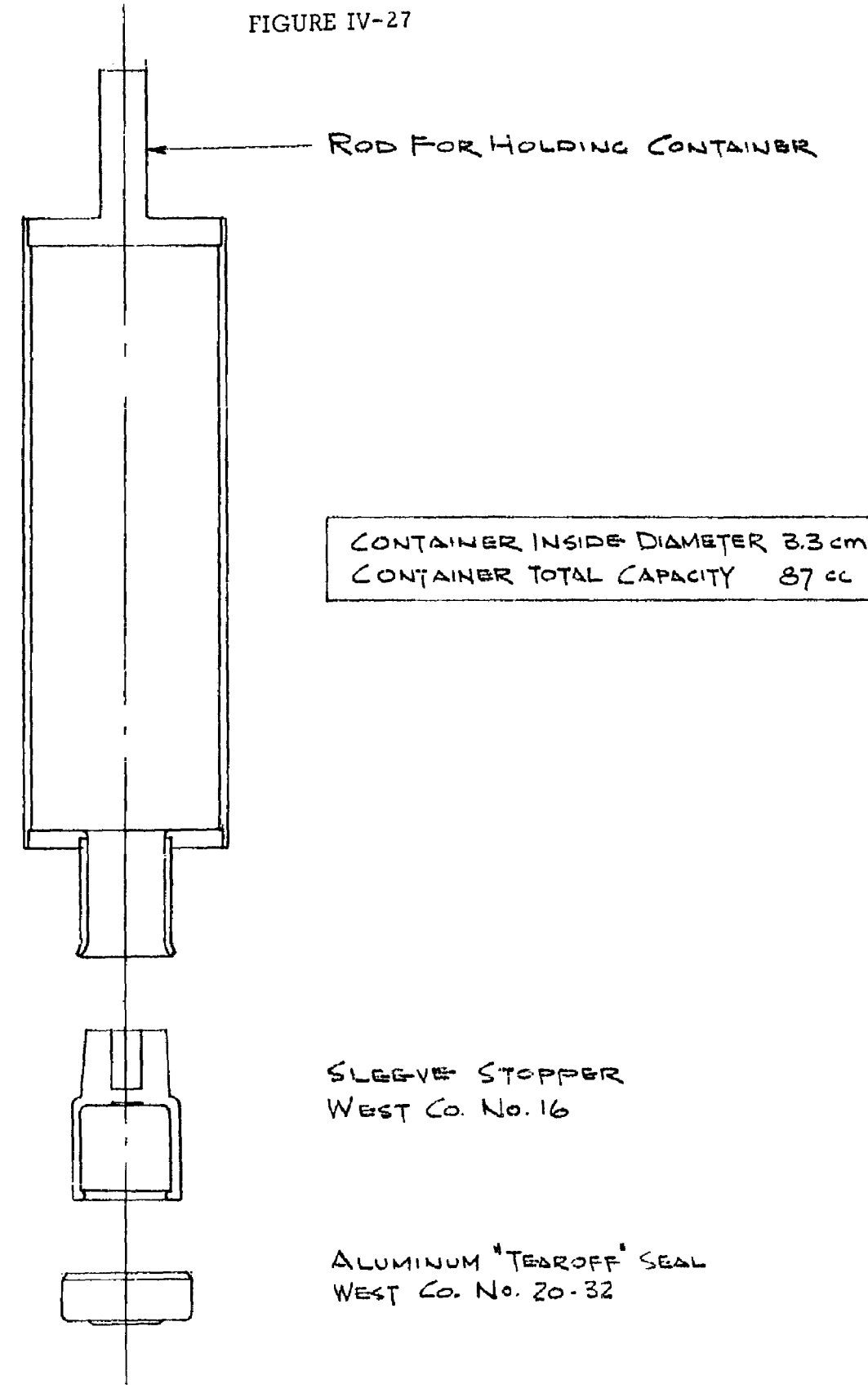


: Mixing and gentle agitation for 30 minutes at room temperature

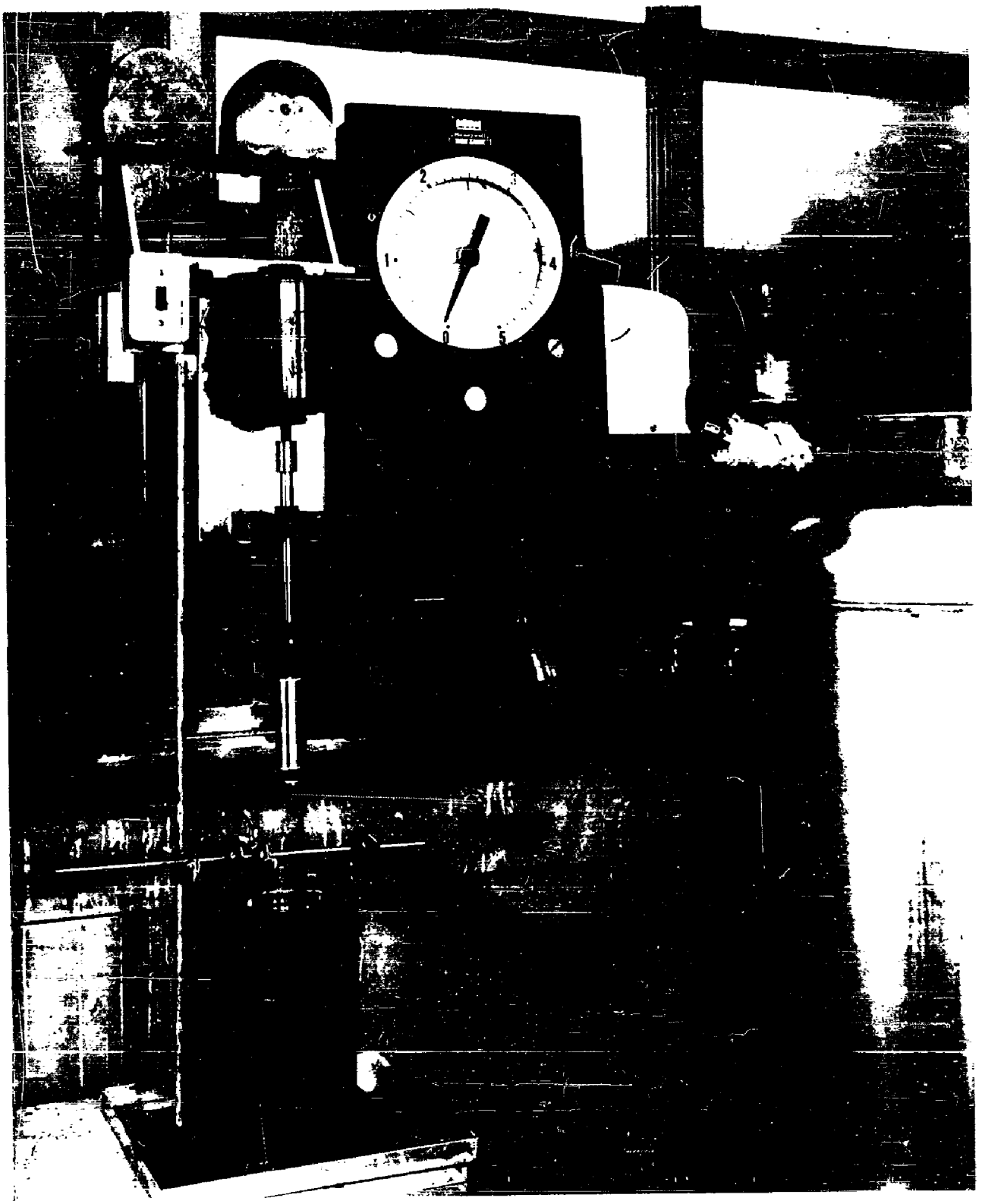
Thawing and Incubation: Stagnant at 45 °C for 2 minutes then 37 °C for 28 min.

Normalization: To 1 volume of thawed preparation add 1/2 volume of saline or 1 volume of plasma.

FIGURE IV-27



EXPERIMENTAL CONTAINER
FOR SPIN FREEZING



Spin-freezing Equipment

FIGURE IV - 28

in Figure IV-29. From these and other experiments we decided to optimize our process using the spin-freeze method. Several reasons for this selection in preference to BPU freezing follow:

1. Maximum cell recoveries are equivalent.
2. Dependence of recovery on initial heat flux is less critical.
3. Volume of nitrogen required to freeze a sample is at least an order of magnitude less.
4. Less risk of mechanical damage to erythrocytes.
5. Good method for scanning chemical variables since freezing variables have only a slight effect on recoveries.
6. If method could be extended to larger volumes (1 pint), necessary equipment would be much simpler and less expensive than BPU-1.

Many types of agitation for the thawing of frozen blood were investigated using Process A, Figure IV-26. Equipment was designed which oscillates a tubular container about its axis through an angle of 80 degrees as well as providing up and down axial agitation, simultaneously or independently of each other. Movement frequencies of up to 275 cycles per minute are possible. With this, and BPU agitation during thawing in the frequency range of 140-200 cycles per minute, stagnant thawing was also studied. The final thawing procedure adopted calls for stagnant thawing for 2 minutes at 45°C followed by a 28 minute exposure to 37°C.

For process optimization in anticipation of studies in human subjects Process B (Figure IV-26) was selected because it presented fewer problems in centrifugation and allowed the saving of the plasma for dilution of the thawed blood. Final results prior to <u>in vivo</u> testing are presented in Table IV-44.

FIGURE IV-29

FREEZING RED CELLS WITH SERUM ALBUMIN ADDITIVE: EFFECT OF INITIAL HEAT FLUX DURING FREEZE CYCLE ON IN VITRO ANALYSIS OF THAWED, NORMALIZED BLOOD

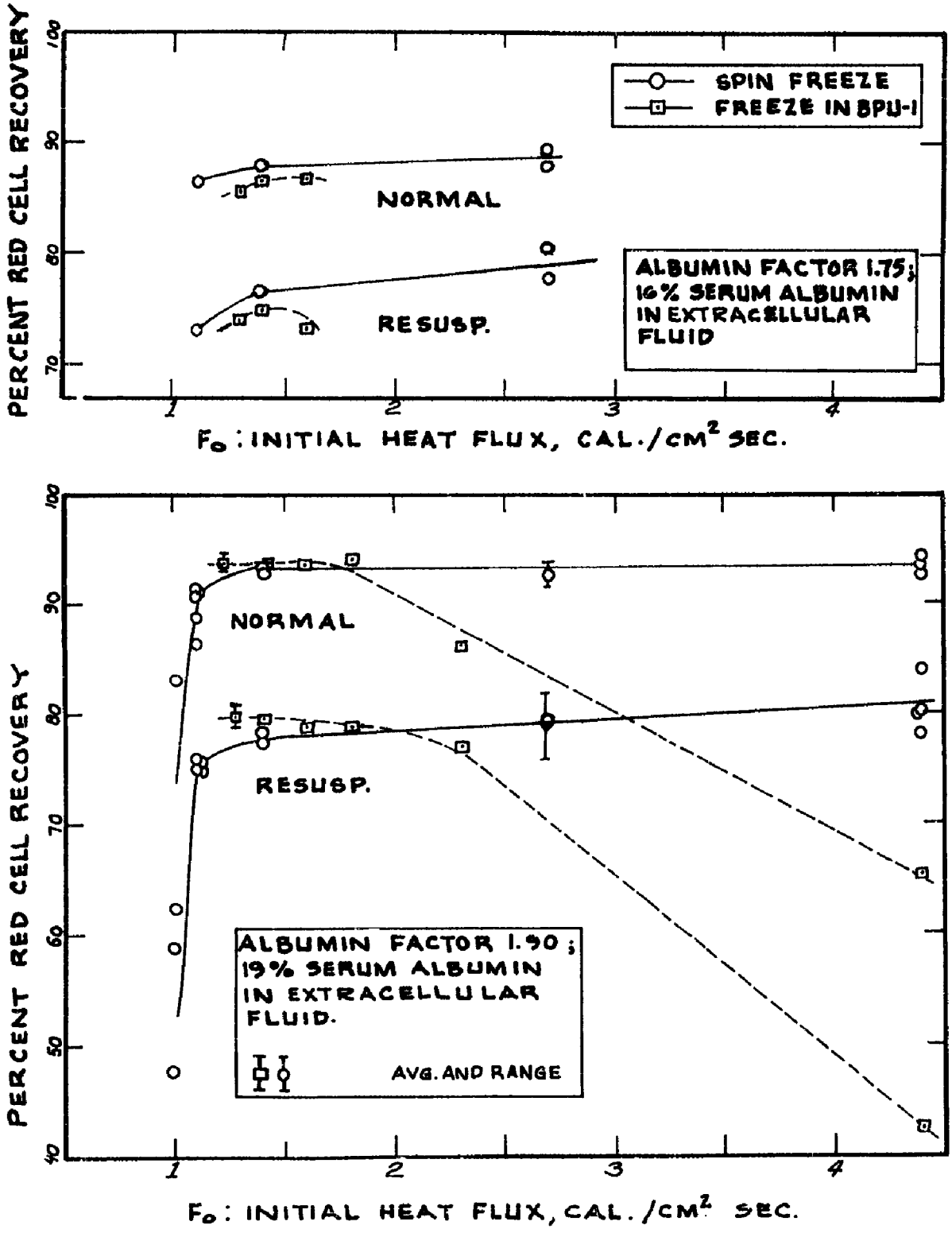


TABLE IV - 44

In-Vitro Recovery of Red Cells Processed by Spin-Freezing with Serum Albumin as Preservative

Sample	Normalized Recovery (%)	Saline Resuspension Recovery (%)
1	92	81
2	93	81
3	93	79
4	93	82
5	94	79
6	92	82
7	92	79
8	92	--
9	92	--
10	92	76
11	94	84
12	93	80
13	93	79

Blood: Drawn into ACD-B and preserved by Process B, Figure IV - 26.

Additive: Solution of 22% serum albumin, 0.6% NaCl. Albumin Factor of 1.9.

Freezing Conditions: 22 cc of packed cells frozen in a 33 mm tubular container. Samples 1-10 frozen in -80 to -100°C liquid and samples 11-13 frozen in liquid nitrogen jets.

Thawing Conditions: Stagnant thaw 2 minutes at 45°C plus 28 minutes at 37°C.

Normalization: 1 volume of plasma added to 1 volume of thawed preparation.

Saline Resuspension: 1 volume of normalized blood diluted to 100 volumes with physiological saline then allowed to stand 1/2 hour prior to analysis for free hemoglobin.

V. RESULTS OF ANIMAL STUDIES

In this section we report the results of animal experiments conducted to determine the viability of red cells after rapid freezing and thawing in the presence of polymeric protective additives. Rabbits were used as the experimental animals, both as donors and recipients. All transfusions involved 5 ml of homologous blood or red cell suspensions; assays were done by consecutive chromium-51 tagging as described previously [9].

Animal tests have provided a basis for assuring the relative safety of subsequent clinical tests, and have allowed a more detailed study of processing variables than would have been possible in human subjects. Except for certain differences in cooling conditions required for optimum recovery and survival in whole blood systems, results obtained in rabbits and in humans with respect to red cell viability have been comparable.

1. Process I: Red Cells in Polymer Medium

Whole blood was taken from donor rabbits by heart puncture into ACD anticoagulant, 1 volume ACD to 3 volumes whole blood. After centrifuging 30 minutes at 2000 RPM, the plasma was removed and stored at -30°C. The red cells were suspended 1:2 in a protective additive solution and frozen in aluminum containers of 19 mm cross-section. Experimental parameters examined included cooling and warming rates, salt concentration, various protective additives, and additive concentration. All samples were shell frozen in liquid nitrogen using 70 second agitation at 200 cycles/minute in the BPU-1 (Blood Processing Unit).

After thawing in the BPU-1 at 200 cycles/minute and 45°C water for 30-35 seconds, samples were centrifuged 30 minutes at 2000 rpm and the red cells were resuspended 1:2 in their original plasma. *In vitro* recoveries were measured immediately after thawing and again after resuspension. Aliquots were labeled with chromium-51 Rachromate (Abbott Laboratories) and *in vivo* viability 1/2 hour and 24 hours after transfusion was estimated by the consecutive chromium-51 procedure [9]. All experiments involved 4-6 rabbits as recipients for 4-6 separate specimens of blood (i.e. each from an individual donor animal). Control experiments demonstrated no alteration in survival by suspension of red cells in polymer solutions and resuspension in plasma without freezing.

Cooling and warming conditions are reported in terms of A/VR ratios as discussed previously in Appendix II of Progress Report III (Rinfret, ONR Progress Report III, 1960). A figure showing the relation of A/VR to volume frozen and heat transfer coating employed is provided in Appendix A of this report.

Type and Concentration of Polymer

Polyvinylpyrrolidone of three average molecular weights and dextran (M. W. 40,000) afforded reasonably effective protection (Table V-1). PVP

TABLE V-1

EFFECT OF TYPE AND CONCENTRATION OF POLYMERIC ADDITIVE

Additive		Salt		% RBC Recovery		% RBC Survival	
Type	Conc.	Conc.	A/VR	Direct	Resp.	1/2 Hr.	24 Hr.
PVP K-30	35%	0.15 M	0.028	96±1	--	71±13	53±10
PVP K-30	35%	0.15 M	0.033	93±2	82±3	86±16	67±2
PVP K-30	20%	0.15 M	0.028	95±1	87±2	81±3	66±5
PVP K-30	20%	0.15 M	0.033	93±1	90±2	74±18	64±12
PVP K-30	15%	0.15 M	0.033	90±2	80±2	84±7	62±6
PVP K-30	15%	0.05 M	0.033	86±2	90±2	82±8	58±10
PVP K-22	15%	0.05 M	0.033	90±2	82±2	77±10	60±5
PVP K-15	35%	0.15 M	0.028	97±1	81±3	80±5	66±7
PVP K-15	20%	0.15 M	0.028	87±3	73±2*	81±3	60±1
PVP K-15	15%	0.15 M	0.028	86±2	80±1	88±2	66±3
Dextran-40	20%	0.15 M	0.028	91±1	88±3	73±7	56±4

*Contaminated plasma; value probably low.

RBCs suspended in an equal volume of additive-saline medium of composition shown and frozen in BFF 1975 or BFF 19110 containers in liquid nitrogen using PVP-Methanol coating and mechanical agitation. Thawing was done with mechanical agitation in 45°C water. Thawed cells were resuspended in autologous plasma prior to transfusion.

Values are averages ±1 S.D. for 4-6 rabbit transfusions.

K-30 (M.W. 40,000) and K-22 (M.W. 25,000) were essentially equivalent. Dextran was less effective than the polyvinylpyrrolidones. Concentration effects (from 35-15%) at best were slight, especially in terms of *in vivo* survival.

Salt Concentration

Variation in salt concentration from 0.025 to 0.15 Molar in the presence of K-30 polyvinylpyrrolidone at 15% concentration was studied. As shown in Table V-2 increasing salt improved direct recoveries. At isotonic (0.15M) concentration, however, resuspension recovery in plasma was decreased. No significant effect of salt concentration on either immediate or 24-hour survival was observed.

TABLE V-2

EFFECT OF SALT CONCENTRATION ON RED CELL RECOVERY AND SURVIVAL

Additive		Salt		% RBC Recovery		% RBC Survival	
Type	Conc.	Conc.	A/VR	Direct	Resp.	1/2 Hr	24 Hr
PVP K-30	15%	0.025M	0.033	72±5	90±1	81±8	67±8
PVP K-30	15%	0.05M	0.033	86±2	90±2	82±8	58±10
PVP K-30	15%	0.15M	0.033	90±2	80±2	84±7	62±6

See footnotes table V-1.

Cooling and Warming Conditions

Relatively slow cooling conditions (A/VR = 0.019) resulted in decreased *in vitro* recoveries (direct and plasma resuspension) and markedly reduced immediate and 24-hour survivals. (Table V-3). Cooling at A/VR ratios from 0.028 to 0.050 by varying volume from about 30% to 50% of container capacity, while using a constant heat transfer coating (PVP-methanol), failed to produce significant differences in recoveries or survival. A slight improvement in 24 hour viability may have resulted from the most rapid cooling and warming conditions examined.

Slight, but not statistically significant, improvement in 24-hour survival resulted from thawing in a 55°C versus 45°C bath.

TABLE V-3

EFFECT OF COOLING AND WARMING CONDITIONS

ON RED CELL RECOVERY AND SURVIVAL

Additive		Salt		Thaw	% RBC Recovery		% RBC Survival	
Type	Conc.	Conc.	A/VR	Temp. °C	Direct	Resp.	1/2 Hr.	24 Hr.
PVP K-30	20%	0.15 M	0.050	45	93±1	91±2	81±21	78±10
PVP K-30	20%	0.15 M	0.033	45	93±1	90±2	74±18	64±12
PVP K-30	20%	0.15 M	0.028	45	95±1	87±2	81±3	66±5
PVP K-30	20%	0.15 M	0.019	45	84±12	77±15	52±13	47±1
PVP K-30	15%	0.05 M	0.033	45	86±2	90±2	82±8	58±10
PVP K-30	15%	0.05 M	0.028	55	93±1	89±1	82±4	67±4

See footnotes to Table V-1. A/VR varied by varying volume frozen.
All containers coated with PVP-methanol except A/VR 0.019 (uncoated).

2. Process II: Red Cells in Plasma-Polymer Medium

Preliminary to clinical study of red cells frozen in medium consisting of polyvinylpyrrolidone in combination with plasma, two animal experiments involving 6 rabbits each were conducted. Results are shown in Table V-4. *In vitro* recoveries and *in vivo* survivals were better than those obtained under optimal conditions using polyvinylpyrrolidone medium alone.

TABLE V-4

RECOVERY AND SURVIVAL OF RABBIT RED CELLS FROZEN IN PLASMA-POLYVINYLPYRROLIDONE

System	% RBC Recovery		% RBC Survival	
	Direct	Resp.	1/2 Hr	24 Hr
RBC-Plasma-PVP	97±0	96±1	84±12	74±8
RBC-Plasma-PVP	96±1	96±1	81±7	74±7

RBC (1 vol.) in 1.5 vols. of mixture of equal parts by volume of autologous plasma and 30% PVP K30 in 0.05M NaCl. Final composition of Medium : 50% plasma-15% PVP-0.1M NaCl. Resuspension 1:2 in 6% dextran-saline.

Container: BFF-19110, coated with PVP-methanol.

Volume Frozen: 50 ml.

Cooling: 200 cycles/min. agitation on BPU-1 in liquid nitrogen, 70 secs.

Warming: 150 cycles/min. agitation on BPU-1 in 45°C water.

This system has been studied further in large volume transfusions in rabbits. Approximately 1/3 the blood volume was withdrawn via heart puncture or ear vein and replaced by frozen homologous red cells in 6% dextran-isotonic saline. Preliminary results demonstrate that whole blood oxygen content, markedly reduced if replacement is by dextran alone, can be effectively restored by the frozen cell suspension. Additional work is in progress in which blood oxygen, plasma hemoglobin, and serum potassium levels are being followed with time after transfusion.

3. Process III: Whole Blood-Polymer Systems

Types and Concentrations of Polymers

Rabbit blood diluted 4:5 with various polymeric compounds in saline was subjected to rapid freezing and thawing. Except in one experiment all containers were coated with Santocel-glycerol during freezing to improve heat transfer. Freezing in all cases was by mechanical shaking on a modified Burrell shaker using liquid nitrogen as refrigerant. Thawing in all cases was by mechanical shaking in water at 45°C.

Aluminum containers 19 mm cross-section were employed, with one exception -- the freezing of blood modified by glycerol pectate which was done in 33 mm cylindrical containers. Containers were of two sizes, 75 ml and 110 ml. Blood-additive mixtures occupied approximately 60% (or 40 ml and 66 ml respectively) of the container's volume.

Specimens froze as a uniform shell covering the inside of the container. Cooling was carried out for 30-45 seconds; warming was for a similar period of time. Storage in the frozen state was at -170 to -196°C for up to about two weeks.

Transfusions were made within 1 to 2 hours after thawing and, except for Cr^{51} tagging at room temperature (30-60 minutes), thawed samples were maintained in an ice bath.

Red cell recoveries and survivals were determined by procedures employed in our assay work. Survival assays employed consecutive tagging and results are therefore relative to the survival of the test animal's own red cells auto-transfused without freezing. Viability at 30-60 minutes could, ideally, be 100%, if no cell injury occurred.

In terms of *in vitro* % red cell recoveries following freezing and thawing several polymers of various molecular weights* afforded effective protection (>85%). Dextran-10 (M. W. 10,000) and dextran-40 (M.W. 40,000) were more effective than dextran-150 (M.W. 150,000). Polyethylene glycols ("Carbowaxes", Union Carbide Chemicals Co.) of M. W.'s of 1,000, 6,000, and 17,500 afforded good protection *in vitro* (80% to 93% recoveries). Glycerol pectate* * and human and bovine serum albumins protected red cells effectively (88%-91%). As reported previously, polyvinylpyrrolidone exhibited effective protective activity. Both M. W.'s of 40,000 (PVP, K-30) and 10,000 (PVP, K-15) allowed high recoveries when added to blood at concentrations of 3.5 to 7% (w/v). Results are summarized in Tables V-5, V-6, and V-7.

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*All molecular weights cited are averages.

** Kindly supplied by Dr. Joseph F. Saunders of the Office of Naval Research.

TABLE V-5

RED CELL RECOVERY AND SURVIVAL OF RABBIT BLOOD

RAPIDLY FROZEN AND THAWED WITH VARIOUS

DEXTRANS AS PROTECTIVE ADDITIVES

Exp. No	No. of Samples	Additive	Average M. W. x 10^{-3}	Conc. % W/V	Container	% RBC Recovery	% RBC Survival 30-60 Min.	24 Hr.
1	6	Dextran-10	10	6	BFF-1975	86±2	73±5	67±10
2	6	Dextran-10	10	6	BFF-1975	89±3	73±6	54±4
3*	5	Dextran-40	40	6	BFF-1975	75±4	52±8	43±8
4	4	Dextran-40	40	6	BFF-1975	93±1	76±7	59±8
5	5	Dextran-150	150	6	BFF-1975	77±5	60±8	47±11

*Frozen without use of Santocel-glycerol coating. All other samples frozen by mechanical shaking on Burrell shaker in liquid nitrogen using Santocel-glycerol coating on containers. All samples thawed in 45°C water with mechanical shaking. Specimens consisted of whole blood (3:1 with ACD anticoagulant) diluted 4:5 with additive in saline to give final concentrations shown.

TABLE V-6

RED CELL RECOVERY AND SURVIVAL OF RABBIT BLOOD

RAPIDLY FROZEN AND THAWED WITH VARIOUS POLYMERS

(POLYETHYLENE GLYCOLS, ALBUMINS, GLYCEROL PECTATE)

Exp. No	No. of Samples	Additive	Conc. % W/V	% RBC Recovery	% RBC Survival 30-60 Min.	% RBC Survival 24 Hr.
6	5	PEG-1000	7	80±2	93±6	87±6
7	5	PEG-6000	7	92±3	65±6	51±5
8	4	PEG-20M	7	93±2	75±6	61±6
9	6	Glycerol Pectate	6	91±1	--	--
10	3	Albumin (HSA)	6	88±2	--	--
11	2	Albumin (BSA)	6	91±1	73±6	54±4

PEG - polyethylene glycols (Union Carbide Chemicals Co., "Carbowaxes" (M. W.'s PEG-1000, 1000; PEG-6000, 6000; PEG-20M, 17,500. All M. W.'s are averages.)

HSA - human serum albumin; BSA-bovine serum albumin

Glycerol pectate samples were frozen in coated BFT-3365 containers; all others were frozen in BFF-1975 containers, coated with Santocel-glycerol and mechanically shaken in liquid nitrogen. Thawing in all cases was with mechanical shaking in 45°C water.

TABLE V-7

RED CELL RECOVERY AND SURVIVAL OF RABBIT BLOOD

RAPIDLY FROZEN AND THAWED WITH POLYVINYLPYRROLIDONE

AS THE PROTECTIVE ADDITIVE

Exp. No	No. of Samples	Additive	Average M. W. x 10^{-3}	Conc. W/V %	% RBC Recovery	% RBC Survival 30-60 Min.	% RBC Survival 24 Hr.
12(1)	6	PVP K-30	40	7	95±1	80±5	64±5
13(2)	5	PVP K-30	40	3.5	84±3	85±11	80±9
14(2)	5	PVP K-30	40	3.5	84±1	76±6	68±9
15(2)	5	PVP K-30	40	1.75	69±2	68±15	68±8
16(1)	5	PVP K-15	10	7	96±1	85±11	73±11
17(1)	5	PVP K-15	10	7	95±1	82±6	74±9
18(1)	6	PVP K-15	10	3.5	91±1	74±9	57±9

PVP - polyvinylpyrrolidone (Oxford Laboratories, K-30; Antara, K-15)

(1) BFF-1975 containers used
(2) BFF-19110 containers used

All samples consisted of blood (3:1 in ACD anticoagulant) diluted 4:5 with additive in saline giving final concentrations shown. All were frozen by mechanical shaking in liquid nitrogen using Santocel-glycerol coatings. Thawing in all cases was by mechanical shaking in water at 45°C.

Red cell viability in vivo following freezing and thawing did not always parallel in vitro recoveries. Survivals at 30-60 minutes post-transfusion ranged from 60 to 93% excluding some preparations frozen with dextran without improved heat transfer coatings.

Reproducibility among replicate experiments was within experimental error (c.f. exp. 1 and 2, Table V-5; exp. 16 and 17, Table V-7). When in vitro recoveries were low, in vivo viability of the recovered red cells was in general reduced significantly (exp. 3 vs 4, Table V-5; exp. 5, Table V-5; and exp. 15, Table V-7).

While significant red cell loss occurred in the circulation between 1 and 24 hours post-infusion, in general, the major loss, up to 25% of the cells infused, occurred within the first 30-60 minutes following transfusion.

Red cell survivals at 24 hours ranged from 47 to 87%, again excluding those dextran preparations frozen less rapidly than other samples. Best results in terms of 24 hours survival (and over-all yield of red cells) were obtained with polyethylene glycol (7% concentration; M.W. 1,000) and polyvinylpyrrolidone K-15 (7% concentration; M.W. 10,000). In both cases over-all yields of 70% (including all in vitro and in vivo losses) were observed 24 hours after transfusion. Polyvinylpyrrolidone (3.5%), dextran-10 (6%), and polyethylene glycol (7%) (M.W. 17,500) gave yields of 50-60%.

Cooling and Warming Conditions

The effects of volume and heat transfer coatings on whole blood-7% PVP (K-30) were studied. Results are summarized in Table V-8. Empirically, optimum conditions can be defined in terms of the variables examined. An adequate explanation of the results obtained is not easily formulated. Since a distinct optimum in survival with regard to volume was obtained for those samples frozen under conditions of a high surface heat transfer coefficient (i.e. 0.04 cal./cm^{2}sec°C), it is possible that a combined effect of cooling rate and warming rate must be considered. Thus, we might conclude that only the most rapid cooling employed was adequate in conjunction with the range of warming rates obtained (as a result of the variation in volumes) to give high viability. The drop at 65 ml would then be ascribed to inadequate warming conditions, while the drop at 45 ml would most likely be ascribed to excessively rapid cooling.

Variation in frequency of mechanical agitation at an amplitude of 2-1/2 inches indicated that shaking can be both too slow and too rapid (Table V-9). Thawing at 35°C or 55°C appeared to be less favorable than thawing at 45°C when using a system of whole blood-7% polyvinylpyrrolidone, otherwise frozen and thawed under apparently optimum conditions of surface heat transfer, volume, and agitation (Table V-10).

TABLE V-8

EFFECTS OF HEAT TRANSFER COATINGS AND VOLUME ON RED CELL RECOVERY AND SURVIVAL IN RAPIDLY FROZEN RABBIT BLOOD

Volume Frozen ml.	% Early Survival Heat Transfer Coating			% 24-Hour Survival Heat Transfer Coating		
	UNC	PVP	SG	UNC	PVP	SG
35±3	66±2	57±5	--	51±2	47±10	--
45±3	58±3	64±6	71±3	45±1	53±6	57±5
55±3	--	56±5	84±5	--	44±7	69±3
65±3	71±13	76±6	67±9	56±3	64±4	54±6

System: Whole Blood (4 vol.) - Polyvinylpyrrolidone (K-30) 35% in isotonic saline (1 vol.).

Cooling Conditions: Varied (Liquid Nitrogen Refrigerant)

Warming Conditions: Thawed 45°C water

Agitation Conditions: 200 cycles/min., 2-1/2-inch amplitude

Container: BFF-19110
PVP - Polyvinylpyrrolidone-Methanol
UNC - Uncoated; SG - Santocel-Glycerol

Each value refers to average of one to six groups of animals. Each group contained 4-6 rabbits.

TABLE V-9

EFFECTS OF CONDITIONS OF AGITATION ON RED CELL RECOVERY AND SURVIVAL IN RAPIDLY FROZEN RABBIT BLOOD

Agitation Frequency CPM		% RBC	% RBC Survival	
Cooling	Warming	Recovery	1/2 Hr.	24 Hr.
100	100	94±2	49±5	39±5
100	200	92±1	71±6	54±8
100	300	76±4	66±11	60±12
200	100	97±1	88±4	71±6
200	200	96±2	82±3	66±6
200	300	80±3	78±8	69±11
300	100	96±4	87±8	71±10
300	200	94±1	71±14	62±9
300	300	71±3	87±5	70±8

System: Whole blood (4 vol.) - polyvinylpyrrolidone (K-30) - 35% in saline (1 vol.)

Cooling Conditions: Liquid nitrogen; Santocel-glycerol coating

Warming Conditions: Water 45°C

Agitation Amplitude: 2-1/2 inch

Container: BFF-19110 Volume: 55 ml

Each value refers to average ±1 S. D. obtained with 4-6 rabbits.

TABLE V-10

EFFECTS OF THAWING TEMPERATURE ON RED CELL RECOVERY

AND SURVIVAL OF RAPIDLY FROZEN RABBIT BLOOD

Thaw Bath Temperature, °C	% RBC Recovery	% RBC Survival 1/2 Hr.	% RBC Survival 24 Hr.
35	95±1	75±7	61±2
35	95±1	80±8	66±11
45	95±1	85±7	71±5
45	95±1	92±14	65±7
45	94±1	85±3	74±2
55	97±1	74±11	68±6
55	97±1	79±5	55±7

System: Whole blood (4 vol.) - polyvinylpyrrolidone (K-30) - 35% in saline (1 vol.).

Cooling: Liquid nitrogen; Santocel-glycerol coating

Warming: Temperatures shown; water bath

Agitation: BPU-1 mechanical agitation 200 cpm. Agitation 2-1/2-inch amplitude on cooling and warming.

Container: BFF-19110 Volume: 55 ml

Each value refers to average ±1 S. D. of 4-6 rabbits.

VI. RESULTS OF CLINICAL STUDIES

Clinical studies of blood preserved at low temperature by processes described in the preceding sections of this report have been carried out at two institutions in Buffalo, New York. Under the direction of Dr. Marvin L. Bloom, Associate Clinical Professor of Medicine, University of Buffalo and Dr. Ernest Witebsky, Distinguished Professor and Chairman of the Department of Bacteriology and Immunology, University of Buffalo School of Medicine, clinical investigations were carried out at the Veterans Hospital, Buffalo, New York. Dr. Charles D. Bull, Internal Medicine Service, and members of the hospital staff (including Serology, Blood Bank, Hematology, Bacteriology, and Radioisotope Service) cooperated in the studies. Additional clinical studies of a research nature, involving only small volume infusions (10 ml) have been carried out by Dr. Raymond S. Kibler, Department of Nuclear Medicine, Roswell Park Memorial Institute, Buffalo, New York.

In this section we describe the results of all clinical studies according to the type of process employed for freezing and thawing. Except where otherwise indicated (i.e. for Process I studies) red cell viability has been measured by consecutive chromium-^{51} tagging as described in Progress Report IX to the Office of Naval Research, with exceptions noted in Appendix C of this report.

1. Process I: Red Cells in Polymer Medium

Clinical studies of Process I have served a several-fold purpose. Most important, a high *in vivo* viability of human red cells subjected to rapid freezing and thawing while protected by a polymeric solute was unequivocally demonstrated. In addition the probable clinical applicability of polymer-protected rapidly frozen red cells in full pint volumes was indicated. Finally, problems associated with a rigorous determination of red cell viability of preserved, especially frozen, red cells in man were defined and resolved.

Results of small volume tests using both P^{32}-Cr^{51} tagging [10] and consecutive Cr^{51} tagging are summarized in Table VI-1. Results are shown graphically in Figures VI-1 and VI-2. Erroneously high red cell survivals were consistently obtained by P^{32}-Cr^{51} tagging under conditions in which radioassay of red cells rather than whole blood was done and washing of the experimental Cr^{51}-labelled cells was omitted. Animal experiments established that the P^{32}-Cr^{51} technique necessitated washing of both control and test cells to avoid erroneously elevated results. Because of an osmotically sensitive population among frozen red cells, application of P^{32}-Cr^{51} with test cell washing would lead to survival assay of a fractionated population of cells. Use of consecutive Cr^{51} tagging avoids this problem.

Results of transfusions of half pint volumes of blood frozen by

TABLE VI-1

Summary of Red Cell Recovery and Survival in Human Subjects

(Small Volume Transfusions)

A. Phosphorus-32 (Control RBCs) - Chromium-51 (Test RBCs)

Controls[a]	% RBC Recovery		% RBC Survival			
	Initial	Resusp.	10 min.	24 hr.	48 hr.	72 hr.
Grand Avg. ± S.D.	-	-	105 ± 8	100 ± 8	96 ± 8	94 ± 8
No. Items	-	-	30	29	27	22
Freeze-Thaw (PVP-LS)[b]						
Grand Avg. ± S.D.	90 ± 4	91 ± 3	107 ± 15	93 ± 18	87 ± 18	82 ± 16
No. Items	43	43	37	36	33	31

B. Chromium-51 (Control RBCs) - Chromium-51 (Test RBCs)

Controls[a]	% RBC Recovery		% RBC Survival			
	Initial	Resusp.	30 min.	24 hr.	48 hr.	72 hr.
Grand Avg. ± S.D.	-	-	95 ± 5	90 ± 6	86 ± 7	84 ± 5
No. Items	-	-	13	16	12	8
Freeze-Thaw (PVP-LS)[b]						
Grand Avg. ± S.D.	90 ± 2	93 ± 3	90 ± 9	74 ± 12	69 ± 11	60 ± 9
No. Items	30	30	28	28	25	15

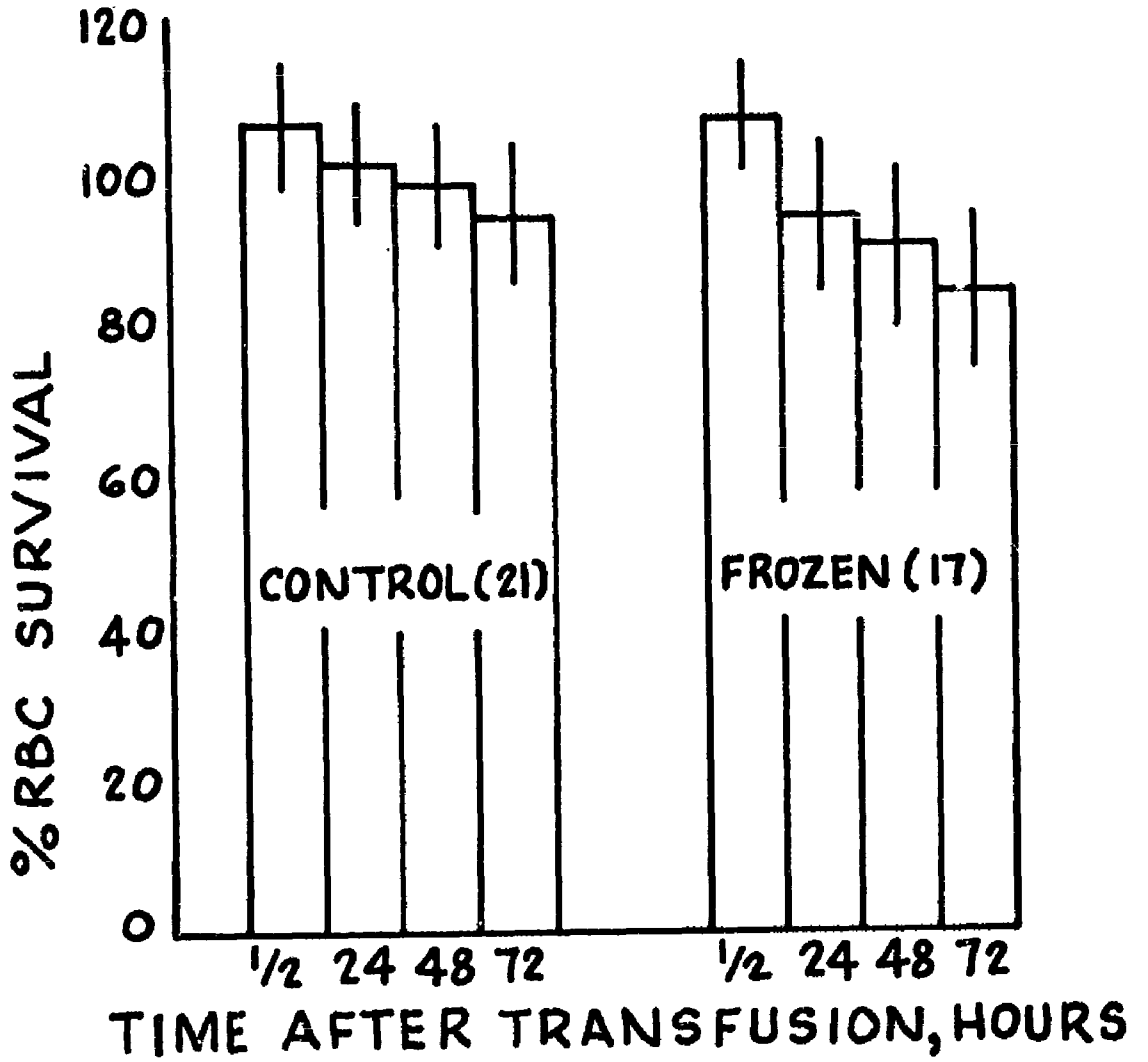
(a) Untreated cells.

(b) RBCS frozen and thawed in 15% PVP-0.05 M NaCl and resuspended in plasma for transfusion.

FIGURE VI-1

SURVIVAL OF CONTROL AND FROZEN RED CELLS MEASURED

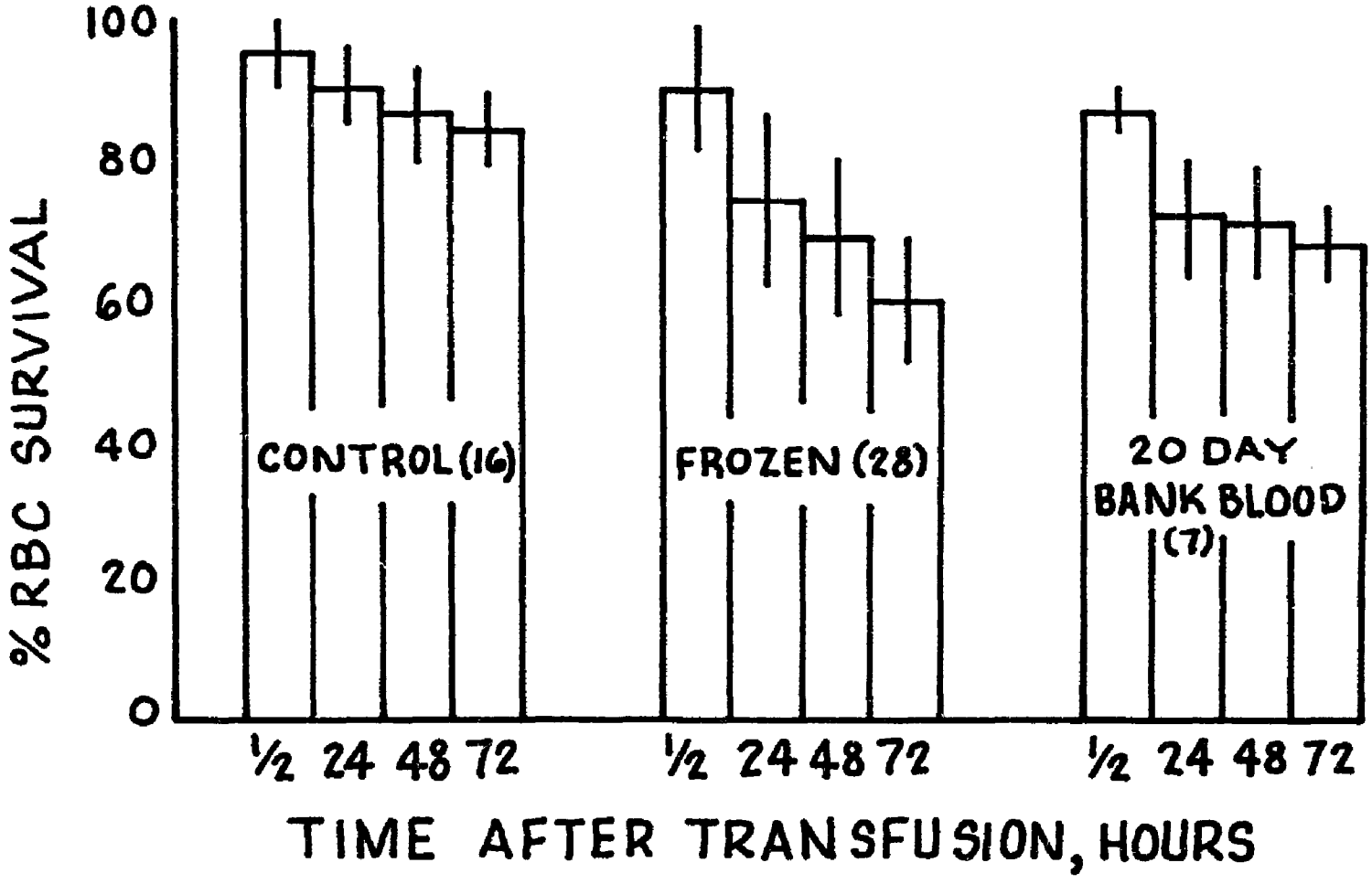
BY PHOSPHORUS-32: CHROMIUM-51 TAGGING



Numbers of tests are indicated in parentheses. Vertical lines within bars indicate ± 1 standard deviation. Frozen blood processed according to Process I.

SURVIVAL OF CONTROL, FROZEN, AND 20 DAY OLD BANK BLOOD

RED CELLS MEASURED BY CONSECUTIVE CHROMIUM-51 TAGGING



Numbers of tests are indicated in parentheses. Vertical lines within bars indicate ± 1 standard deviation. Frozen blood processed according to Process -I.

FIGURE VI-2

Process I are shown in Table VI-2. Similar results for full pints are shown in Table VI-3.

2. Process II: Red Cells in Plasma-Polymer Medium

Process II has been studied clinically in a series of small volume (10 ml) transfusions reported in Tables VI-5 through VI-10. Immediate (10-30 minute) and 24 hour survival for 10 untreated control samples and 5 samples processed without freezing and thawing are shown in Table VI-4. Precision has been approximately ±4% in all cases. Accuracy appears to be very high as evidenced by control cell survival of 99±4% immediately following infusion as compared with the theoretical level of 100%.

For Process II, optimum conditions in terms of red cell survival appear to require metal containers for freezing. Thawing at 37°C was better by several % than thawing at 25° or 45°. Polyvinylpyrrolidone concentration between 15 and 25%, plasma concentration between 15 and 50%, and salt concentration between 0.055 and 0.10 molar afford essentially equivalent preparations on the basis of in vivo red cell survival. Dilution and heat transfer conditions studied appeared to affect slightly immediate postinfusion survival, but not 24-hour survival.

3. Process III: Whole Blood-PVP

Results of direct transfusions in half pint and full-pint volumes of whole blood frozen with 7% polyvinylpyrrolidone w/v are shown in Tables VI-11 and VI-12.

4. Process IV: Reduced Volume System

Small volume studies of the survival of red cells frozen in reduced volume systems with K-30 and K-22 PVP and with human serum albumin are shown in Table VI-13. While the additive systems used show promise of eventual usefulness, recoveries and survivals are inferior, however, to those obtained by the other processes studied.

TABLE VI-2

Summary of Half-Pint Results

Conditions	No. Items	% Recovery		% Survival[b]			
		Direct	Resp.[a]	1/2 hr.	24 hr.	48 hr.	72 hr.
Frozen Proc. I, 15% PVP-LS*	8	88 ± 2	94 ± 1	98 ± 13	86 ± 13	79 ± 12	74 ± 11
Frozen Proc. I, 20% PVP-LS*	8	88 ± 2	90 ± 2	87 ± 9	73 ± 8	67 ± 11	68 ± 8
Frozen Proc. I, 20% PVP-NS*	7	88 ± 4	88 ± 3	85 ± 8	75 ± 9	73 ± 7	71 ± 9
Frozen Proc. II**	3	89	90	89	79	73	73
Controls, unfrozen fresh	17	-	-	103 ± 7	97 ± 8	92 ± 8	90 ± 9
Controls, 4-day, 4°C. storage	3	-	-	105	103	98	97

Standard deviations shown as ± values after each average value.

*Equal volumes RBCs and additive solution: LS = 0.05 M NaCl
NS = 0.15 M NaCl.

** Process II = equal parts plasma and 30% PVP; approximately 2 vol. plasma-PVP medium used for each volume RBCs.

a. Resuspension recovery = % recovery following resuspension of thawed cells in plasma.

b. Survival by consecutive Chromium-51 procedure; % of transfused RBCs.

TABLE VI-3

Red Cell Survival Following Transfusion of

Full Pints of Blood Frozen by Process I

Transf. No.	Volume Transfused (ml.)	Direct RBC Recovery %	Resuspension Recovery %	% RBC Survival 30 min.	24 hr.	48 hr.	72 hr.
A. 15% PVP-LS							
1	456	91	84	72	66	62	50
2	493	88	80	67	50	53	39
3	505	92	91	55	51	49	36
4	451	93	91	60	48	55	39
Avg. ± S.D.		91 ± 2	86 ± 5	63 ± 8	54 ± 8	55 ± 5	41 ± 6
Control-A1	522	-	-	103	97	98	82
Control-A2	518	-	-	106	100	101	93
Avg.				104.5	98.5	99.5	87.5
B. 20% PVP-NS							
5	343	96	94	74	71	61	62
6	300	96	93	78	70	71	69
7	284	96	96	85	64	66	65
Avg. ± S.D.		96 ± 0	94 ± 2	79 ± 6	68 ± 4	66 ± 5	65 ± 4
C. 20% PVP-LS							
8	451	95	95	94	82	76	76
9	460	95	95	94	73	82	72
10	485	95	96	84	73	68	71
11	371	94	94	87	76	72	74
12	378	94	94	95	88	81	82
13	-	95	96	-	-	-	-
Avg. ± S.D.		95	95 ± 1	91 ± 5	78 ± 7	76 ± 6	75 ± 4
Control C1	501	-	-	106	103	99	98
Control C2	508	-	-	76	76	72	74
Avg.				91	89	75	86

TABLE VI-3 Cont'd

Transf. No.	Volume Transfused (ml.)	Direct RBC Recovery %	Resuspension Recovery %	% RCB Survival 30 min.	24 hr.	48 hr.	72 hr.
D. 20% PVP-LS							
14	470	96	94	77	71	62	60
15	443	97	97	81	62	58	50
16	436	95	92	70	64	58	58
17	437	95	91	69	58	52	51
Avg. ± S.D.		96 ± 1	94 ± 3	74 ± 6	64 ± 5	57 ± 4	55 ± 5
Control D1	544	-	-	110	94	82	74
Control D2	514	-	-	94	82	76	71
Avg.				102	88	79	72

Equal volumes of red blood cells combined with additive solutions indicated, and frozen in 1100-ml. capacity container. Thawed by agitation in 45°C. water bath. Thawed cells resuspended in autologous plasma.

Controls: Untreated autologous blood infused not more than 24 hours after withdrawal.

LS: 0.05 M NaCl
NS: 0.15 M NaCl

Note: Section D: Blood collected and handled in Fenwal plastic bags.

TABLE VI-4

CONTROL STUDIES OF RED CELL SURVIVAL

Conditions	No. Tests	% RBC Recovery		% RBC Survival	
		Direct	Resusp.	10-30 Min.	24 Hr.
Untreated Blood[a]	10	--	--	99±4	91±4
Prefreeze Handling[b]	5	99	100	105±3	97±3

(a) Autologous blood, collected by venipuncture, labeled with Cr^{51} (1/2 hr) and reinfused.

(b) Processed according to Process-II (in 50% Plasma-15% PVP-0.1M NaCl) - including all transfers, shaking (200 cpm), resuspension in dextran, etc., but without freezing and thawing.

TABLE VI-5

EFFECT OF THAWING BATH TEMPERATURE ON RED CELL VIABILITY (PROCESS II)

Thaw Bath Temp. °C	No. Tests	% RBC Recovery		% RBC Survival	
		Direct	Resusp.	10-30 Min.	24 Hrs.
45	3	96±0	97±1	88±6	71±5
37	6	94±2	97±1	93±6	78±5
25	3	86±6	96±1	87±1	69±3

System: RBCs in 50% Plasma-15% PVP-0.1M NaCl.
Dilution: RBC/Total volume = 1/2.6
Conditions: 50 ml in BFF-19110 container coated with PVP-MeOH. Mech. agitation in liquid N_2 200 cycles/min (cpm), 2-1/2 in. amplitude. Thawed at 25-45°C, 150 cpm.

All values are average ±1σ. Resuspensions in 6% Dextran.

TABLE VI-6

EFFECTS OF HEAT TRANSFER COATING AND TYPE OF CONTAINER ON RED CELL VIABILITY (PROCESS II)

Container	Cross Section mm	Heat Transf. Coating	No. Tests	% RBC Recovery Direct	% RBC Recovery Resusp	% RBC Survival 10-30 min	% RBC Survival 24 Hrs
Metal	19	PVP-Methanol	2	96±2	96±1	93±3	74±3
Metal	19	None	2	97±1	98±0	84±2	72±1
Plastic	25	None	3	96±1	98±1	78±5	59±6

System: RBCs in 50% Plasma-20% K30 PVP-0.075M NaCl.
Dilution: RBC/total vol.= 1/2.6.
Conditions: Cooling in liquid N_2 200 cycles/min, 2-1/2 in. ampl.
Warming 37°C water, 150 cycles/min, 2-1/2 in. ampl.

All values are averages ± 1σ. Resuspensions in 6% Dextran.

TABLE VI-7

EFFECT OF DILUTION

Dilution Factor RBC/Total	No. Tests	% RBC Recovery Direct	% RBC Recovery Resusp.	% RBC Survival 10-30 Min.	% RBC Survival 24 Hrs.
1/2.5-2.8	3	96±0	97±1	88±6	71±5
1/4.5-4.7	3	96±0	96±1	92±3	74±3

System: RBCs in 50% Plasma - 15% PVP-0.1M NaCl.
Conditions: 45-90 ml in BFF-19110 metal containers with PVP-MeOH coating. Mech. agitation in liquid N_2 200 cpm, 2-1/2 in. ampl. Thawed in 45°C water at 150 cpm, 2-1/2 in. ampl.

All values averages ± 1σ. Resuspensions in 6% Dextran.

TABLE VI-8

EFFECT OF PLASMA CONCENTRATION ON RED CELL VIABILITY (PROCESS II)

System	No. Tests	% RBC Recovery Direct	% RBC Recovery Resusp.	% RBC Survival 10-30 Min	% RBC Survival 24 Hr.
50% Plasma - 20% PVP - 0.075 M NaCl	2	96±2	96±1	93±3	74±3
30% Plasma - 20% PVP - 0.075 M NaCl	2	95±3	98±1	90±4	76±1
15% Plasma - 20% PVP - 0.075 M NaCl	3	95±1	97±1	88±1	78±4

Dilution: RBCs/Total Volume = 1/2.6
Conditions: 50 ml in BFF-19110 containers coated with PVP-MeOH.
Mech. agitation in liquid N_2, 200 cpm, 2-1/2 in. ampl.
Thawed in 37°C water, 150 cpm.

All values are averages ± 1 σ. Resuspensions in 6% Dextran.

TABLE VI-9

EFFECTS OF CONCENTRATION OF POLYVINYLPYRROLIDONE ON RED CELL VIABILITY (PROCESS II)

System	No. Tests	%RBC Recovery Direct	%RBC Recovery Resusp.	% RBC Survival 10-30 Min	% RBC Survival 24 Hr
30% Plasma - 10% PVP - 0.06 M NaCl	1	88	91	76	62
30% Plasma - 15% PVP - 0.06 M NaCl	1	88	94	74	77
30% Plasma - 20% PVP - 0.06 M NaCl	1	92	95	86	76
30% Plasma - 25% PVP - 0.06 M NaCl	1	96	95	92	78
30% Plasma - 30% PVP - 0.06 M NaCl	1	97	93	81	72

Dilution: RBC/Total Volume = 1/2.6
Conditions: 50 ml in BFF-19110 containers coated with PVP-MeOH.
Mech. agitation in liquid N_2, 200 cpm, 2-1/2 in. ampl.
Thawed in 37°C water, 150 cpm.

All values in this table are single transfusions. Resuspensions in 6% Dextran.

TABLE VI-10

EFFECT OF SALT CONCENTRATION ON RED CELL VIABILITY (PROCESS II)

System	No. Tests	% RBC Recovery		% RBC Survival	
		Direct	Resusp.	10-30 Min.	24 Hrs.
30% Plasma-20% PVP-0.055M NaCl	1	92	97	93	77
" " -0.06M NaCl	1	92	95	86	76
" " -0.075M NaCl	1	93	98	92	76
" " -0.085M NaCl	1	97	97	87	75

Dilution: RBC/Total volume = 1/2.6

Conditions: 50 ml in BFF-19110 containers coated with PVP-MeOH. Mechanical agitation in liquid N_2, 200 cpm, 2-1/2 in. ampl. Thawed in 37°C water, 150 cpm.

All values in this table are single transfusions. Resuspensions in 6% Dextran.

TABLE VI-11

Red Cell Survival Following Transfusion of Blood

Frozen in Half-Pint Volumes by Process III

Transf. No.	Volume Transfused (ml.)	in vitro % RBC Recovery	% RBC Survival 30 min.	24 hr.	48 hr.	72 hr.
1	285	97	-	-	-	-
2	292	97	78	76	72	72
3	287	96	81	81	74	76
4	171	96	99	-	87	-
5	164	97	103	86	91	80
6	166	96	95	86	76	70
7	151	97	96	88	88	85
8	293	98	86	-	73	75
9	290	98	95	-	82	-
10	298	97	97	-	84	82
Avg. ± S.D.		97 ± 1	92 ± 9	83 ± 5	81 ± 7	77 ± 5
11*	139	91	77	70	56	-
Control 1	281	-	93	88	84	82
Control 2	258	-	106	100	-	97
Control 3	289	-	101	94	88	88
Control 4	239	-	98	-	95	89
Avg. ± S.D.			99 ± 5	94 ± 6	89 ± 6	89 ± 6

*Lowered recovery resulting from incomplete thawing of the frozen preparation in the blood processing unit.

Whole blood (4 vol.) combined with Plasdone-C (K30 PVP) 0.05 M NaCl (1 vol.) to final concentration of 7% PVP in mixture. Freezing in XFC-1/2 containers. Thawed by agitation in 45°C. water bath; transfused without post-thaw processing.

Controls: Untreated autologous blood.

Note: Whole blood was collected directly into Plasdone-C-ACD-B anticoagulant in the freeze-thaw container for Transfusions 8-10.

TABLE VI-12

RED CELL SURVIVAL FOLLOWING TRANSFUSION OF BLOOD

FROZEN IN FULL PINT VOLUMES BY PROCESS III

Transf. No	Volume Transfused ml	In Vitro RBC % Recovery	mg% Plasma Hemoglobin 30 Min. Post-Transfusion	% RBC Survival 30 Min.	24 Hr.	48 Hr.	72 Hr.
1	386	97	68	87	60	57	54
2	390	95	49	83	59	55	55
3	381	96	91	81	64	63	60
Average	386	96	69	84	61	58	56
Control-2	258	--	1	103	94	94	90
4	--	97	--	--	--	--	--
5	377	96	59	99	83	78	78
6	382	97	30	--	85	75	74
7	379	98	35	98	86	79	78
8	383	97	48	97	82	74	77
Average ±S. D.	380	97	43	98±1	84±2	77±2	77±2
Control-1	290	--	2	103	97	92	85

Whole blood (4 vol.) combined with Plasdone-C (K-30 PVP) 0.05M NaCl (1 vol.) to final concentration of 7% PVP in mixture. Frozen in 1100 ml capacity containers. Thawed by agitation in 45°C water bath. Transfused without post-thaw processing.

Controls: Untreated autologous blood.

Note: The relatively poor survival values obtained with the first group of three subjects require comment. While difficulties were encountered with donor scales, which prevented accurate proportioning of whole blood and PVP solution, the 30 minutes, post-transfusion, plasma hemoglobin levels do not indicate a red cell loss even approximating an average 16%. Prior work suggests that early red cell loss is hemolytic in nature. Thus, a rough calculation, based on an assumed circulating plasma volume of 3 liters, would indicate that abrupt lysis of an average of 16% of the transfused cells should have given an average plasma hemoglobin level of about 250 mg% in these experiments. The failure to find this suggests a fault in assay procedure, or, a very rapid, non-hemolytic clearance of about 15% of the transfused cells from the circulation. In the latter case, no explanation consistent with the findings in the next group of five subjects is at hand.

TABLE VI-13

Red Cell Recovery and Survival for Process IV

Conditions	Thaw Temp. °C.	No. Tests	% RBC Recovery After Dilution	% RBC Survival 10-30 min.	% RBC Survival 24 hr.
Proc. IV (Plasma-K30 PVP)[a]	45	4	88 ± 3	88 ± 3	73 ± 3
Prov. IV (Plasma-K30 PVP)[a]	37	2	90 ± 0	86 ± 4	68 ± 6
Proc. IV (Plasma-K22 PVP)[b]	37	3	94 ± 2	83 ± 4	71 ± 3
Proc. IV (Albumin System)[c]	45	2	92 ± 0	74 ± 5	54 ± 4

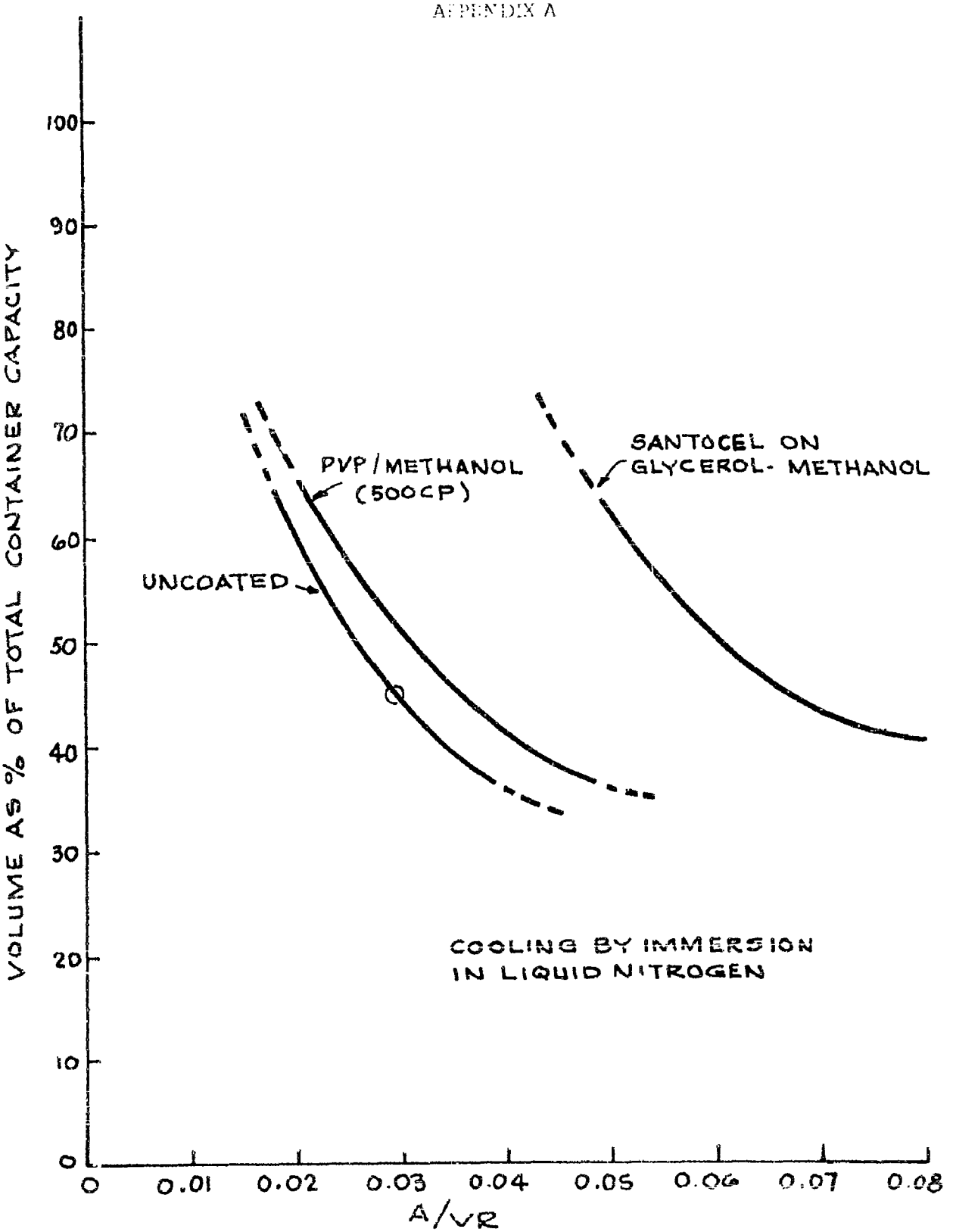
a. Whole blood mixed with 30% K30 PVP-0.05 M NaCl equal to plasma volume, centrifuged, and supernatant removed leaving a volume of suspending medium equal to 1/4 the cell volume. Frozen as for Process II. Thawing done with agitation at 45°C. or manually at 37°C. Samples diluted with isotonic NaCl (3 vol. saline per 5 vol. concentrated RBC-additive mixture).

b. Same as (a) except that 30% K22 PVP-0.05 M NaCl was used.

c. Cell fraction separated from whole blood and combined with 22% human serum albumin-0.6% NaCl (containing total albumin equivalent to 1.9 x that of the original plasma removed). Albumin concentration 19% and hematocrit 56-58% at time of freezing. Frozen in 33-mm. diameter cylindrical containers by spinning mechanically in -100°C. bath. Thawed mechanically at 45°C. and incubated 1/2 hr. at 37°C. Diluted by addition of plasma (1 vol.) to each 2 vol. of thawed blood.

All survivals by consecutive Chromium-51 procedure.

APPENDIX A



A/VR AS A FUNCTION OF VOLUME FROZEN

APPENDIX B

SURVIVAL CURVES FOR FROZEN AND UNFROZEN RED CELLS

Curves showing individual recipient values for red cell survival in three experiments are included in this appendix.

Figures 1 and 2 are survival curves obtained using P^{32}-Cr^{51} labeling. Excessively high levels of survival and high degrees of variance are illustrated. The general reduction in rate of red cell disappearance per day after 24-48 hours is seen for the frozen preparations.

Figure 3 shows typical results obtained with the same frozen blood process using consecutive Cr^{51} tagging to estimate survival.

FIGURE B - 1

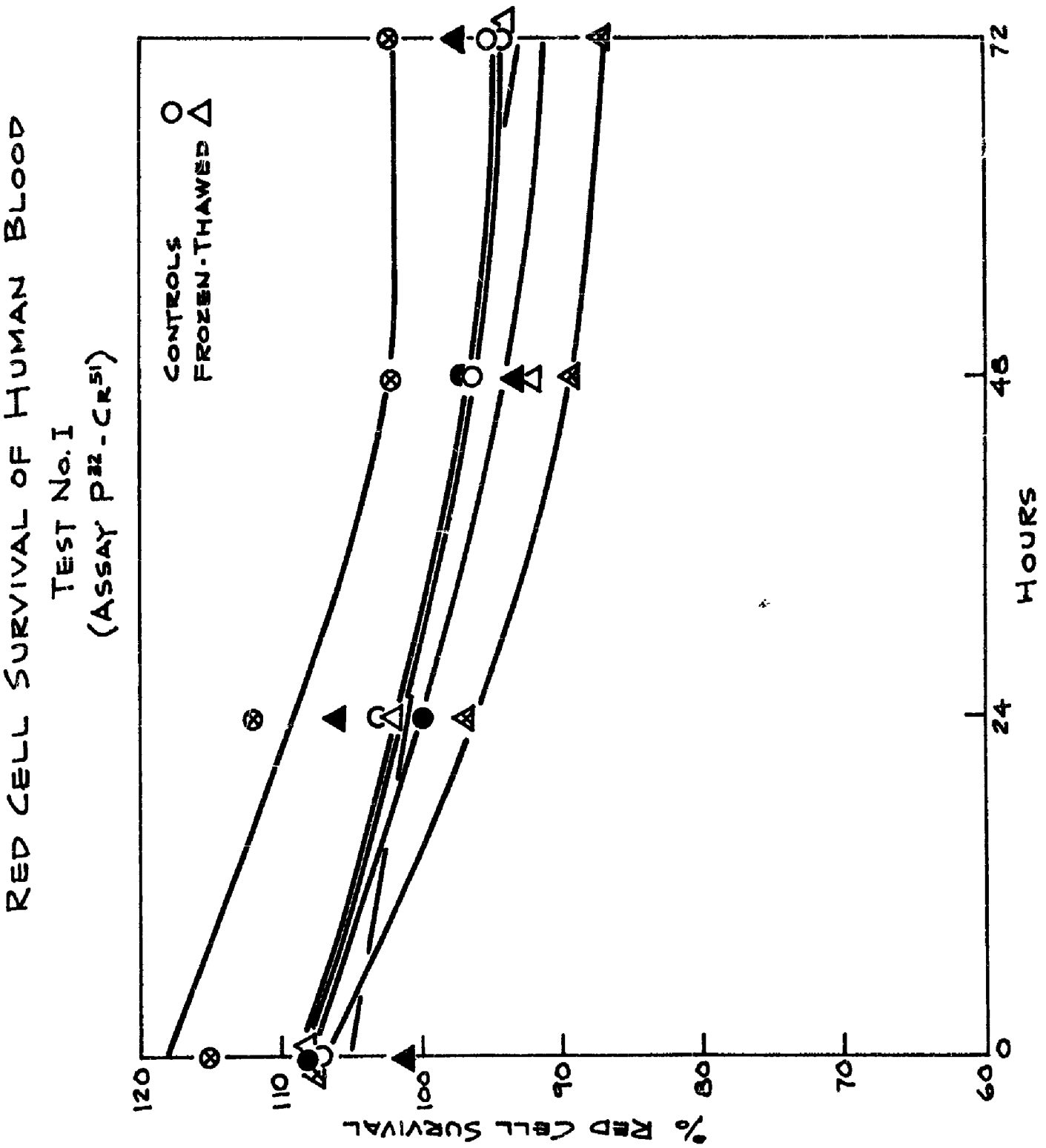

RED CELL SURVIVAL OF HUMAN BLOOD
TEST No. I
(ASSAY P32-CR51)
CONTROLS
FROZEN-THAWED
% RED CELL SURVIVAL
120
110
100
90
80
70
60
0
24
48
72
HOURS

FIGURE B - 2

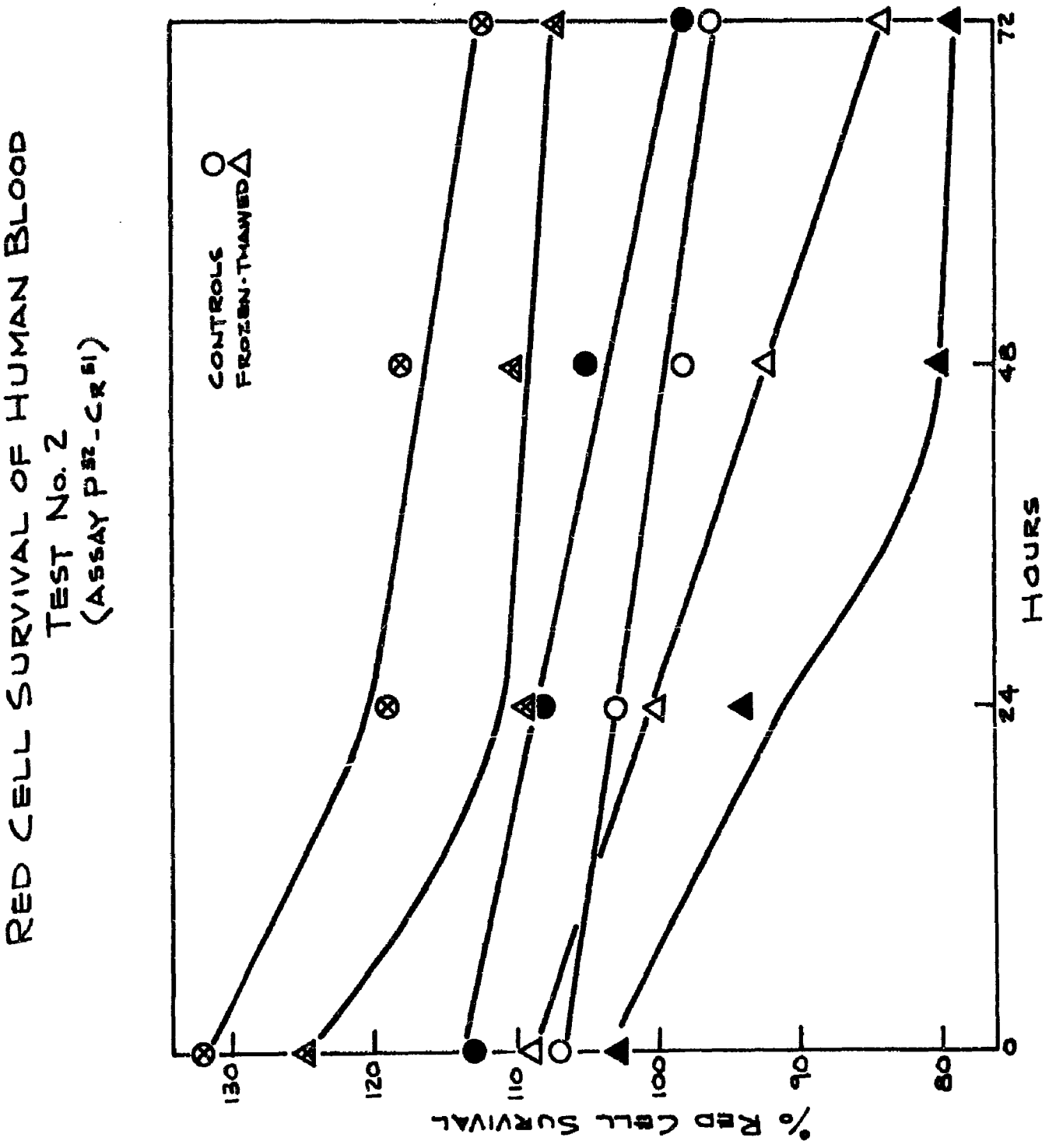
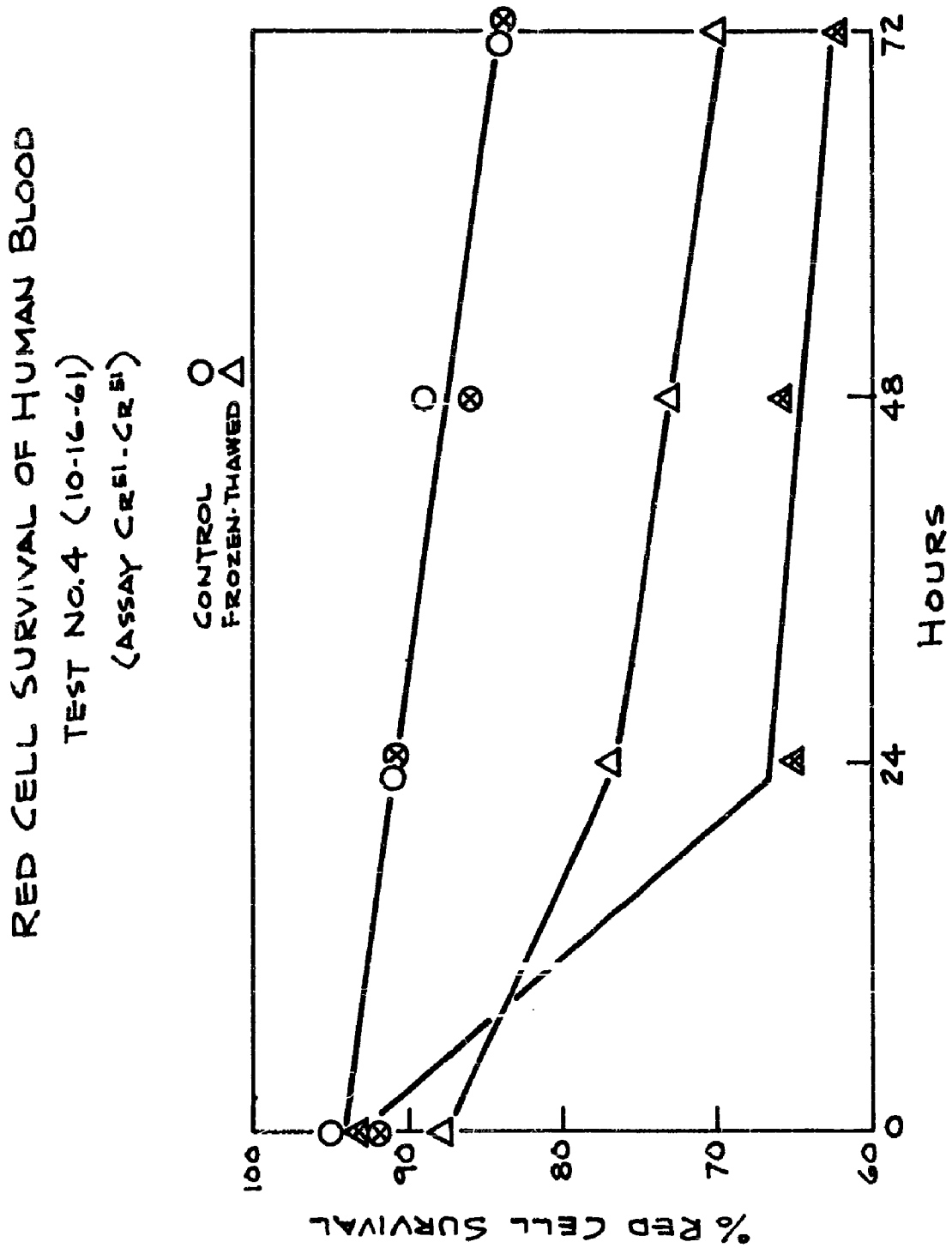

RED CELL SURVIVAL OF HUMAN BLOOD
TEST No. 2
(ASSAY P^{32}-Cr^{51})
CONTROLS
FROZEN-THAWED
% RED CELL SURVIVAL
130
120
110
100
90
80
0
24
48
72
HOURS

FIGURE B - 3


RED CELL SURVIVAL OF HUMAN BLOOD
TEST NO.4 (10-16-61)
(ASSAY CR51-CR51)
CONTROL
FROZEN-THAWED
% RED CELL SURVIVAL
100
90
80
70
60
0
24
48
72
HOURS

APPENDIX C

DETERMINATION OF RED CELL SURVIVAL IN MAN BY CONSECUTIVE CHROMIUM-51 TAGGING

The procedure of survival estimation by consecutive Cr^{51} tagging in man is identical to that previously described for animals (ONR Progress Report IX, 1961) with the following exceptions:

1. All injections and samples are made into and taken from an arm vein. Opposite arms are used for injection and sampling.

2. Volumes of 10 to 15 ml are employed for the S and T injections. For large volume transfusions (i.e. 1/2-1 pint volumes) quantities infused are measured by weight.

3. A total of 25 μc chromium-51 is injected for the T-injection.

4. Except for large volume transfusions (i.e. > 100 ml) correction for red cell volume change during a test need not be applied. In any event correction for sample volumes (approximately 10 ml each) need not be applied.

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